REPÚBLICA ARGENTINA
PODER EJECUTIVO NACIONAL
MINISTERIO de ECONOMÍA y PRODUCCIÓN
SECRETARÍA de INDUSTRIA, COMERCIO y de la PEQUEÑA y MEDIANA EMPRESA
INSTITUTO NACIONAL de la PROPIEDAD INDUSTRIAL

# CERTIFICADO DE DEPÓSITO

ACTA N° P 19990100681

LA ADMINISTRACIÓN NACIONAL DE PATENTES, CERTIFICA QUE CON FECHA 5 DE FEBRERO DE 1999 SE PRESENTO A NOMBRE DE BIO SIDUS S.A.; CON DOMICILIO LEGAL EN ALSINA 971 1° PISO OF. "10" - BUENOS AIRES -, REPUBLICA ARGENTINA (AR).

UNA SOLICITUD DE PATENTE DE INVENCIÓN RELATIVA A: PROCEDIMIENTO DE CULTIVO MASIVO DE CELULAS DE MAMIFERO PARA LA OBTENCION DE ERITROPOYETINA HUMANA RECOMBINANTE Y LA ERITROPOYETINA HUMANA RECOMBINANTEOBTENIDA CON TAL PROCEDIMIENTO

CUYA DESCRIPCIÓN Y DIBUJOS ADJUNTOS SON COPIA FIEL DE LA DOCUMENTACIÓN DEPOSITADA EN EL INSTITUTO NACIONAL DE LA PROPIEDAD INDUSTRIAL.

SE CERTIFICA QUE LO ANEXADO A CONTINUACIÓN EN 54 FOJAS ES COPIA FIEL DE LOS REGISTROS DE LA ADMINISTRACIÓN NACIONAL DE PATENTES DE LA REPÚBLICA ARGENTINA DE LOS DOCUMENTOS DE LA PATENTE DE INVENCIÓN PRECEDENTEMENTE IDENTIFICADA.

A PEDIDO DEL SOLICITANTE, EXPIDO LA PRESENTE CONSTANCIA DE DEPOSITO EN BUENOS AIRES, REPUBLICA ARGENTINA, A LOS 13 DÍAS DEL MES DE ABRIL DE 2005.

DR. EDUARDO ARIAS
COMISARIO
ADMINISTRACION NACIONAL DE PATENTES

**CERTIFIED COPY OF PRIORITY DOCUMENT**

INSTITUTO NACIONAL DE LA PROPIEDAD INDUSTRIAL
ARGENTINA



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PUBLICA ARGENTINA

I.N.P.I.

SOLICITUD DE:

PATENTE DE INVENCION: [x]

CERTIFICADO DE MODELO DE UTILIDAD: [ ]

RR681 23-2-99 01

Dr. EDUARDO R. ARIAS
Representante Legal
Administración Nacional de Patentes

Fecha de Presentación

I. Solicitante

Acta Nº:

1) Apellido y Nombre / Denominación o Razón Social:

**BIO SIDUS S.A.**

2) Documento de Identidad:

Estado Civil:

Nupcias:

Nombre del Cónyuge:

3) Caja de Jubilación o AFJP: C.U.I.T.

Nº de CUIL o CUIT: 30-59811709-4 IVA: RESPONSABLE INSCRIPTO

4) Inscripto en el Registro Industrial de la Nación (Decreto-Ley 19.971/72) Nº

5) Domicilio Real: CONSTITUCION 4234 - BUENOS AIRES - ARGENTINA

Legal: ALSINA 971 - 1º piso, of. "10" - BUENOS AIRES

II. Objeto

6) Título de la Invención: "PROCEDIMIENTO DE CULTIVO MASIVO DE CELULAS DE MAMIFERO PARA LA OBTENCION DE ERITROPOYETINA HUMANA RECOMBINANTE Y LA ERITROPOYETINA HUMANA RECOMBINANTE OBTENIDA CON TAL PROCEDIMIENTO"

7) Carácter de la Patente:

a) Definitiva, por el término de VEINTE años

b) Adicional a la Patente Nº

8) Ley 17.011 Fecha Prioridad:

País

Nº

INSTITUTO NACIONAL DE LA PROPIEDAD INDUSTRIAL
SOLICITUD DE: PATENTE DE INVENCION TRAMITE: ----------
FECHA: 23/02/1999 HORA: 00:00
RESP: COLL. ARECO, CARLOS MIGUEL
CODIGO DE BARRAS DEL EXPEDIENTE
0199901006812302000O

P19990100681
BUSQUEDA COLA TRABAJO
P19990100681

III. Documentación acompañada

9) Se acompaña:

a) Comprobante pago servicio requerido [ ]

b) Formulario anexo en duplicado [ ]

c) Carátula en duplicado [ ]

AAAPI

d) Memoria descriptiva en duplicado ☐
e) Reivindicaciones en duplicado firmadas ☐
f) 2 copias de la 1º reivindicación ☐
g) Dibujos en triplicado ☐
h) Número de planchas ☐
i) Reducciones ☐
j) Copia certificada (Ley 17.011) ☐
k) Documento de Cesión ☐
l) Dibujos informales ☐

02

IV. Sociedades

10) Sociedad, representada por HUMBERTO MARIO DE PASQUALE

quién declara bajo juramento que inviste el carácter de APODERADO

que su mandato se encuentra vigente y que la Sociedad se halla inscripta en

Fecha 7/10/1983 Nº 7258 Fº -- Lib. 98 Tº A

V. Mandato

11) Poder inscripto en: _____ Registrado en el INPI bajo Nº: _____

_____ Otro Registro: _____ Nº: _____

12) En este caso, se autoriza a: CARLOS MIGUEL COLL ARECO y/o

HUGO EDUARDO MARTINEZ LAHITOU

para tramitar este asunto hasta su terminación con facultades para firmar documentos, desistir si fuere menester y solicitar testimonios.

13) Se acompaña poder: ☐

14) Caja Jubilación o AFJP CONSOLIDAR Nº CUIL o CUIT: CUIT 20-04991729-6 / CUIT 20-16821007-9

15) Agente Nº: 611/ 900

VI. Declaración

16) A los efectos del Decreto sin número del 7 de Junio de 1901 (sobre patentabilidad en el extranjero) manifiesta que el invento NO ha sido patentado en el extranjero.

VII. Observaciones SE DECLARA QUE LAS COPIAS AUTENTICADAS DEL CORRESPONDIENTE PODER, ACTA DE CONSTITUCION, DE ASAMBLEA Y DE DIRECTORIO SE ENCUENTRAN AGREGADAS EN LA SOLICITUD DE PATENTE DE INVENCION Nº P98 01 05609

CARLOS MIGUEL COLL ARECO

(Firma del autorizado)

HUMBERTO MARIO DE PASQUALE
APODERADO

(Firma del solicitante)

23 FEB 1999

# Memoria Descriptiva de la Patente de Invención

Sobre

Procedimiento de cultivo masivo de células de mamífero para la obtención de Eritropoyetina Humana Recombinante y la Eritropoyetina Humana Recombinante obtenida con tal procedimiento.

Solicitada por

**Bio Sidus S.A.**

Por el plazo de 20 años

Constitución 4234 - 1254
Buenos Aires - Argentina

# PROCEDIMIENTO DE CULTIVO MASIVO DE CÉLULAS DE MAMÍFERO PARA LA OBTENCIÓN DE ERITROPOYETINA HUMANA RECOMBINANTE Y LA ERITROPOYETINA HUMANA RECOMBINANTE OBTENIDA CON TAL PROCEDIMIENTO

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## I. Descripción Técnica de la Invención

Método para el cultivo masivo de células de mamífero recombinantes para la producción de Eritropoyetina (EPO) humana recombinante. La EPO obtenida mediante el método descrito.

## II. Campo Técnico de la Invención

La presente invención se refiere a un método para el cultivo masivo de células de mamífero recombinantes productoras de EPO que utiliza un medio de cultivo que incluye insulina.

## III. Estado de la Técnica

La EPO es una glicoproteína que estimula la diferenciación de eritroblastos en la médula ósea, incrementando así el número de eritrocitos en la sangre. La vida promedio de los eritrocitos en humanos es de 120 días, por lo cual un ser humano pierde 1/120 de sus eritrocitos cada día. Esta pérdida debe ser contínuamente repuesta para mantener estable la cantidad de glóbulos rojos.

La existencia de la EPO fue postulada desde principio de siglo y fue definitivamente demostrada por Reissman y Erslev a principios de los 50'. Ver Carnot, et al., *C.R. Acad. Sci.*, (Francia), 143, 384-6 (1906); Carnot, et al., *C.R. Acad. Sci.*, (Francia), 143, 432-5 (1906); Carnot, et al., *C.R. Soc. Biol.*, 111, 344-6 (1906); Carnot,

*C.R. Soc. Biol.*, 111, 463-5 (1906); Reissman, *Blood*. 1950, 5, 372-80 (1950) y Erslev, *Blood*, 8, 349-57 (1953). Los experimentos de Reissman y Erslev fueron rápidamente confirmados por otros investigadores. Ver Hodgson, et al., *Blood*, 9, 299-309 (1954); Gordon, et al., *Proc. Soc. Exp. Biol. Med.*, 86, 255-8 (1954) y Borsook, et al., *Blood*, 9, 734-42 (1954).

La individualización del sitio de producción despertó un gran debate. Sucesivos trabajos llevaron a identificar al riñón como el principal órgano y a las células intersticiales peritubulares como el sitio de síntesis. Ver Jacobson, et al., *Nature*, 179, 633-4 (1957); Kuratowska, et al., *Blood*, 18, 527-34 (1961); Fisher, *Acta Hematol.*, 26, 224-32 (1961); Fisher, et al., *Nature*, 205, 611-2 (1965); Frenkel, et al., *Ann. N.Y. Acad. Sci.*, 149, 1, 292-3 (1968); Busuttil, et al., *Proc. Soc. Exp. Biol. Med.*, 137, 1, 327-30 (1971); Busuttil, *Acta Haematol.*, (Suiza), 47, 4, 238-42 (1972); Erslev, *Blood*, 44, 1, 77-85 (1974); Kazal, *Ann. Clin. Lab. Sci.*, 5, 2, 98-109 (1975); Sherwood, et al., *Endocrinology*, 99, 2, 504-10 (1976); Fisher, *Ann. Rev. Pharmacol. Toxicol.*, 28, 101-22 (1988); Jelkmann, et al., *Exp. Hematol*, 11, 7, 581-8 (1983); Kurtz, et al., *Proc. Natl. Acad. Sci.*, (EE.UU.), 80, 13, 4008-11 (1983); Caro, et al., *J. Lab. Clin. Med.*, 103, 6, 922-31 (1984); Caro, et al., *Exp. Hematol*, 12, 357 (1984); Schuster, et al., *Blood*, 70, 1, 316-8 (1986); Bondurant, et al., *Mol. Cell. Biol.*, 6, 7, 2731-3 (1986); Bondurant, et al., *Mol. Cell. Biol.*, 6, 7, 2731-3 (1986); Schuster, et al., *Blood*, 71, 2, 524-7 (1988); Koury, et al., *Blood*, 71, 2, 524-7 (1988); Lacombe, et al., *J. Clin. Invest.*, 81, 2, 620-3 (1988); Koury, et al., *Blood*, 74, 2, 645-51 (1989).

Una proporción menor, de 10% a 15% del total de la EPO, es producida por el hígado en adultos. Ver Naughton, et al., *J. Surg. Oncol.*, 12, 3, 227-42 (1979); Liu, et

al., *J. Surg. Oncol.*, 15, 2, 121-32 (1980); Dornfest, et al., *Ann. Clin. Lab. Sci.*, 11, 1, 37-46 (1981); Dinkelaar, et al., *Exp. Hematol.*, 9, 7, 796-803 (1981); Caro, et al., *Am. J. Physiol.*, 244, 5 (1983); Dornfest, et al., *J. Lab. Clin. Med.*, 102, 2, 274-85 (1983); Naughton, et al., *Ann. Clin Lab. Sci.*, 13, 5, 432-8 (1983); Jacobs, et al., *Nature*, 313, 6005, 806-10 (1985); Erslev, et al., *Med. Oncol. Tumor. Pharmacother.*, 3, 3-4, 159-64 (1986). La EPO se produce proporcionalmente al grado de hipoxia de los tejidos, y su expresión crece mediante el aumento del número de células productoras.

La EPO es una proteína que ha demostrado gran eficacia para el tratamiento de anemias causadas por diferentes factores, en especial la anemia de origen renal. Sin embargo, su disponibilidad terapéutica estuvo limitada hasta hace poco tiempo por la falta de un método de producción masivo, ya que la cantidad y calidad de la EPO obtenida por cualesquiera de los sistemas extractivos conocidos eran insuficientes. Recientemente, el uso de técnicas de ADN recombinante ha viabilizado la obtención de proteínas en grandes cantidades. La aplicación de estas técnicas a células eucarióticas ha permitido la producción a gran escala de EPO. Ver patentes EE.UU. 5.688.679 (Powell), 5.547.933 (Lin), 5.756.349 (Lin), 4.703.008 (Lin) y 4.677.195 (Hewick et al.)

A pesar de existir abundante literatura referente a la producción de EPO en cultivo de células de mamífero, no se ha descrito método alguno que lleve a la producción eficiente de esta proteína en cantidades masivas. Otras de las desventajas de los medios y sistemas de cultivo conocidos son su falta de reproducibilidad y la producción de EPO de baja calidad. Ver patentes EE.UU. 5.688.679 (Powell), 5.547.933 (Lin), 5.756.349 (Lin), 4.703.008 (Lin) y 4.677.195 (Hewick et al.); Andersen, et al., *Curr. Op. Biotech.*, 5, 546-549 (1994); Butler, Ed., "Mammalian Cell Biotechnology",

(IRL Press, Oxford, England, 1991); Murakami, Ed., "Trends in Animal Cell Culture Technology", (Kodansha Ltd., Tokyo, Japan, 1990); Freshney, Ed., Animal Cell Culture. A Practical Approach, Ch. 3, (IRL Press, Oxford, England, 1986); Pirt, "Principles of Microbe and Cell Cultivation", (Blackwell Scientific Pub., London, England, 1985); Hames et als., "Transcription and Translation. A Practical Approach", (IRL Press, Oxford, England, 1984)

IV. Descripción de la Invención

La presente invención describe un método de cultivo de células recombinantes que permite la producción masiva de EPO con las características necesarias para su aprovechamiento industrial en medicina humana. El novedoso método de esta invención permite obtener una producción inesperadamente alta de EPO con bajo contenido de proteínas contaminantes en el medio de cultivo. El bajo nivel de contaminantes facilita la posterior purificación de EPO, lo que resulta en altos porcentajes de recuperación de la proteína. Ello se logra mediante la suplementación del medio de cultivo con insulina.

Otras ventajas adicionales del método son: 1) su reproducibilidad al aumentarse la escala de producción y, 2) la altísima calidad de la EPO producida a todos los niveles de producción.

El procedimiento a que se refiere la presente patente de invención consiste en la producción masiva de EPO a través del uso de células recombinantes conservadas congeladas en nitrógeno líquido (Master y Working Bank). Posteriormente, se descongelan las células y a través de sucesivas expansiones, a 37 °C con distintos medios de cultivo, se alcanza un número de células adecuado para la producción

industrial masiva. Las células se cultivan en frascos T25 (25 $cm^2$ de superficie) y se tranfieren subsecuentemente a frascos de mayor superficie hasta llegar a envases tipo "roller" de 850 $cm^2$ cada uno. El medio de cultivo utilizado para la expansión celular se sustituye entonces por un medio de cultivo mejorado para iniciar la producción de EPO. Luego de un período de tiempo, se recolecta el medio de cultivo conteniendo EPO y se procede a su análisis. Los ejemplos que siguen describen detalladamente la invención reivindicada.

**EJEMPLO 1. CULTIVO**

El microorganismo productor utilizado fue una línea de células de mamífero (CHO) transfectada con el ADN genómico de la eritropoyetina humana. Este clon de células productoras se almacenó congelado en nitrógeno líquido en los respectivos bancos de células ("Master" y "Working Bank"). El congelamiento se hizo siguiendo métodos usuales de esta tecnología. (Ver Hames et al., "Transcription and Translation. A Practical Approach", 1984).

Posteriormente, se descongelaron 4 "semillas" del clon productor, provenientes del "Working Bank" donde se preservaron congeladas en $N_2$ líquido y cada una de las "semillas" se cultivó en fase sólida estacionaria (Frascos T 25) en 10 ml de medio de cultivo Núm. 1 (Ver Tabla 1). Para cada Frasco T25 se procedió como sigue:

El cultivo celular se desarrolló a 37 °C, durante 24 h en atmósfera con 5 % de $CO_2$. Posteriormente se cambió el medio de cultivo, permaneciendo las células en 10 ml de medio Núm. 2 (Ver Tabla 1), a la misma temperatura y porcentaje de $CO_2$ durante otras 24 h. Luego, se tomó el frasco T 25 con células recombinantes productoras de EPO y se llevó a cabo una sucesión de etapas de expansión que se describe en los siguientes

09

ejemplos:

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**EJEMPLO 2. EXPANSIÓN 1**

Las células obtenidas en el ejemplo anterior se despegaron del frasco T25 donde crecieron, mediante el tratamiento con solución de tripsina siguiendo protocolos de uso común en cultivo celular, (Ver Hames et al., "Transcription and Translation. A Practical Approach", 1984) y se sembró 20 % de las células en cada uno de 5 frascos T25, que contenían 10 ml de medio de cultivo Núm. 2 (Ver Tabla 1). Las células se cultivaron durante 48 horas a 37 ° C.

**EJEMPLO 3. EXPANSIÓN 2**

Las células obtenidas en la expansión 1 se despegaron de los frascos T25 donde crecieron, mediante el tratamiento con solución de tripsina siguiendo protocolos de uso común en cultivo celular, y se sembraron las células de cada T 25 en un frasco T 150, con 75 ml de medio de cultivo Núm. 1 (Ver Tabla 1). Las células se cultivaron entonces durante 72 horas a 37 ° C.

**EJEMPLO 4. EXPANSIÓN 3**

Las células obtenidas en la expansión 2 se despegaron de los frascos T150 donde crecieron, mediante el tratamiento con solución de tripsina siguiendo protocolos de uso común en cultivo celular. Se sembró 1/10 de las células provenientes de cada T 150 en un nuevo frasco T 150, con 75 ml de medio de cultivo Núm. 1 (Ver Tabla 1). Las células se cultivaron entonces durante 72 horas a 37 ° C.

**EJEMPLO 5. EXPANSIÓN 4**

Las células obtenidas en la expansión 3 se despegaron de los frascos T150 donde crecieron, mediante el tratamiento con solución de tripsina siguiendo protocolos de uso

común en cultivo celular. Posteriormente, se sembraron las células provenientes de cada T 150 en un frasco "roller" de 850 $cm^2$ de superficie interior, con 200 ml de medio de cultivo Núm. 1 (Ver Tabla 1). Las células se cultivaron entonces durante 72 horas a 37 °C.

**EJEMPLO 6. COSECHA DE EPO**

La etapa productiva propiamente dicha comenzó con la siembra de células en frascos "roller" (850 $cm^2$ de superficie) con las células provenientes del paso de la expansión 4, en 200 ml de medio de cultivo Núm. 1 repitiendo la metodología antes descrita. Con las células provenientes de cada frasco de la etapa de expansión 4 se cultivaron 15 frascos "roller", o sea, un total de 3.000 frascos "roller" de acuerdo a las siguientes condiciones:

1. Medio de cultivo Núm. 1
2. Tiempo de cultivo: 72 hs
3. Concentración de gases: atmósfera natural
4. Temperatura: 37 °C.
5. Velocidad de giro del "roller": 5,5 minutos

Luego de verificar la formación de la monocapa celular, mediante observación por microscopio invertido, se descartó el medio de cultivo, se lavaron las células con solución Hank's y se continuó el cultivo en medio Núm. 3.

Posteriormante se procedió cada 48 horas a la cosecha del sobrenadante de cultivo de cada "roller" y a su reposición bajo estrictas condiciones de esterilidad. Este proceso se repitió por lote de producción en 5 oportunidades.

Los sobrenadantes cosechados se concentraron 100 veces utilizando un sistema

de filtración tangencial a través de membranas con un corte de peso molecular de 3.000 D, (Amicon S10Y3). El concentrado se esterilizó por filtración y se conservó a - 20 °C. Finalmente, se procedió a reconcentrar el "pool" de fracciones previamente concentradas utilizando un sistema de ultrafiltración tangencial similar al anterior; este último concentrado fue esterilizado por filtración a través de membranas con poros de 0,22 µm de diámetro.

La solución estéril así obtenida proveniente del cultivo de células recombinantes, tiene una alta concentración de EPO y baja proporción de contaminantes.

La densidad celular alcanzada por $cm^2$ resultó de aproximadamente 180.000 células, la viabilidad de las mismas osciló entre el 95 y 98 % a lo largo de todo el procedimiento. El volumen total de medio cosechado fue de 2.900 litros. El concentrado de acuerdo al ejemplo 6 rindió un volumen de 29,5 litros.

Los valores obtenidos por cosecha en el material concentrado fueron:

| Cosecha | EPO-RIE (mg/ml) | EPO-RIE (g) | Prot. Tot. (mg/ml) | Prot. Tot. (g) |
|---|---|---|---|---|
| 1 | 0,90 | 26,55 | 1,74 | 51,33 |
| 2 | 1,29 | 38,05 | 3,25 | 95,87 |
| 3 | 1,28 | 37,76 | 3,39 | 100.0 |
| 4 | 1,10 | 32,45 | 4,57 | 134,8 |
| 5 | 1,12 | 33,04 | 5,48 | 161,7 |
| TOTAL | | 167,85 | | 543,7 |

Posteriormente se procedió a la purificación de la EPO obtenida, recuperándose el 30 % (medido por actividad biológica *in vivo),* rendimiento sorprendentemente alto, que se debe al bajo nivel de impurezas presente en el sobrenadante de cultivo.

Una muestra de la EPO obtenida utilizando el proceso descrito fue primeramente purificada y luego sometida a los análisis siguientes para demostrar su identidad y pureza:

1. En un gel desnaturalizante de poliacrilamida (SDS-PAGE) la EPO purificada observó una banda ancha de más de 30 kDa de peso molecular. Ver Fig. 1.
2. La EPO producida fue reconocida por un anticuerpo monoclonal así como por un anticuerpo policlonal contra EPO humana en un ensayo "Western Blot". Ver Fig. 2.
3. El tratamiento con glicanasas de la EPO producida probó la existencia de las cadenas glicosídicas en cantidad y peso molecular acorde a lo esperado para EPO. Ver Fig. 3.
4. La EPO producida mostró estar compuesta por una serie de especies o isoformas de punto isoeléctrico comprendido entre 3.0 y 4.5. Ver Fig. 4.
5. La secuenciación completa de aminoácidos de la proteína aislada y purificada a partir del sobrenadante de cultivo de las líneas celulares transfectadas mostró total homología con la EPO humana natural que posee la siguiente secuencia de 165 aminoácidos.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| NH2– | Ala | Pro | Pro | Arg | Leu | Ile | Cys | Asp | Ser | Arg |
| | Val | Leu | Glu | Arg | Tyr | Leu | Leu | Glu | Ala | Lys |
| | Glu | Ala | Glu | ***Asn*** | Ile | Thr | Thr | Gly | Cys | Ala |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Glu | Hys | Cys | Ser | Leu | Asn | Glu | ***Asn*** | Ile | Thr |
| Val | Pro | Asp | Thr | Lys | Val | Asn | Phe | Tyr | Ala |
| Trp | Lys | Arg | Met | Glu | Val | Gly | Gln | Gln | Ala |
| Val | Glu | Val | Trp | Gln | Gly | Leu | Ala | Leu | Leu |
| Ser | Glu | Ala | Val | Leu | Arg | Gly | Gln | Ala | Leu |
| Leu | Val | ***Asn*** | Ser | Ser | Gln | Pro | Trp | Glu | Pro |
| Leu | Gln | Leu | Hys | Val | Asp | Lys | Ala | Val | Ser |
| Gly | Leu | Arg | Ser | Leu | Thr | Thr | Leu | Leu | Arg |
| Ala | Leu | Gly | Ala | Gln | Lys | Glu | Ala | Ile | Ser |
| Pro | Pro | Asp | Ala | Ala | ***Ser*** | Ala | Ala | Pro | Leu |
| Arg | Thr | Ile | Thr | Ala | Asp | Thr | Phe | Arg | Lys |
| Leu | Phe | Arg | Val | Tyr | Ser | Asn | Phe | Leu | Arg |
| Gly | Lys | Leu | Lys | Leu | Tyr | Thr | Gly | Glu | Ala |
| Cys | Arg | Thr | Gly | Asp–COOH | | | | | |

***X*** sitios de glicosilación

6. La presencia de los cuatro sitios de glicosilación sobre la cadena de 165 aminoácidos, así como la estructura compleja de hidratos de carbono, y fundamentalmente los residuos de ácido siálico terminales, fueron demostrados conjuntamente con su correcta actividad biológica *in vivo* en el modelo de ensayo del ratón policitémico ex-hipóxico, exhibiendo total paralelismo frente al estándar internacional correspondiente.

La ausencia de suero fetal bovino u otros componentes de origen animal, permite

obtener EPO con un nivel de pureza inusualmente elevado, de aproximadamente el 30%. Mediante el uso de insulina se evita el agregado de otras proteínas, que contaminarían el producto final.

Los métodos de cultivo tradicionales, que utilizan 10 % de suero fetal bovino, producen EPO con una pureza inferior al 1 %, treinta veces menos que mediante el proceso reivindicado.

**TABLA NÚMERO 1**

A. Medio de Cultivo Núm. 1

Medio Base + Suero Fetal Bovino 10 %.

| | | | | | |
|---|---|---|---|---|---|
| ISCOVE's DMEM | 8,85 | g/litro | Triptofano | 27 | mg/litro |
| HAM F12 | 5,35 | g/litro | Asparagina | 40 | mg/litro |
| $NaHCO_3$ | 2,10 | g/litro | Serina | 80 | mg/litro |
| Glucosa | 1,30 | g/litro | Etanolamina | 3 | ml/litro |
| Lactosa | 0,20 | g/litro | Glutamina | 1,90 | g/litro |
| Galactosa | 0,20 | g/litro | Suero Fetal Bovino | 100 | ml/litro |
| Piruvato de Na | 0,11 | g/litro | | | |

B. Medio de Cultivo Núm. 2

Medio Base + Suero Fetal Bovino 10 % + Geneticin 0,5 mg/ml

| | | | | | |
|---|---|---|---|---|---|
| ISCOVE's DMEM | 8,85 | g/litro | Triptofano | 27 | mg/litro |
| HAM F12 | 5,35 | g/litro | Asparagina | 40 | mg/litro |
| $NaHCO_3$ | 2,10 | glitro | Serina | 80 | mg/litro |
| Glucosa | 1,30 | g/litro | Etanolamina | 3 | ml/litro |
| Lactosa | 0,20 | g/litro | Galactosa | 0,20 | g/litro |
| Piruvato de Na | 0,11 | g/litro | Suero Fetal Bovino | 100 | ml/litro |
| Glutamina | 1,90 | g/litro | Geneticina | 500 | mg/litro |

C. Medio de Cultivo Núm. 3

Medio Base + Insulina

| | | | | | |
|---|---|---|---|---|---|
| ISCOVE's DMEM | 8,85 | gr/litro | Triptofano | 27 | mg/litro |

| | | | | | |
|---|---|---|---|---|---|
| HAM F12 | 5,35 | g/litro | Asparagina | 40 | mg/litro |
| $NaHCO_3$ | 2,10 | g/litro | Serina | 80 | mg/litro |
| Glucosa | 1,30 | g/litro | Etanolamina | 3 | ml/litro |
| Lactosa | 0,20 | g/litro | Insulina | 10 | mg/litro |
| Galactosa | 0,20 | g/litro | Piruvato de Na | 0,11 | g/litro |
| Glutamina | 1,90 | g/litro | | | |

D. Solución HANK's

| | | | | | |
|---|---|---|---|---|---|
| $Ca\ Cl_2 . 2H_2O$ | 185 | mg/litro | $Na_2HPO_4$ | 47,8 | mg/litro |
| $MgSO_4 . 7\ H_2O$ | 140 | mg/litro | Glucosa | 1,0 | g/litro |
| ClK | 400 | mg/litro | $NaHCO_3$ | 350 | mg/litro |
| $KH_2PO_4$ | 60 | mg/litro | Rojo Fenol | 11 | mg/litro |
| NaCl | 8,0 | g/litro | | | |

V. Descripción de Diagramas

La Fig. 1 ilustra un análisis en gel de poliacrilamida (SDS-PAGE) de una muestra de la EPO obtenida según el método descrito. En las calles 1, 4 y 7 se ven los marcadores de peso molecular. En las calles 2, 3, 5 y 6 se corrieron diferentes masas de EPO pura obtenida según el proceso reivindicado. Puede apreciarse la pureza del producto obtenido y su peso molecular aparente de algo más de 30 kDa que coincide con el de la EPO humana urinaria.

La Fig. 2 ilustra un análisis "Western Blot" de una muestra de la EPO obtenida según el método descrito. Se verifica la identidad de la EPO producida, ya que es reconocida por un anticuerpo antiEPO humana. En la calle 1 se corrió un estándar de EPO humana, en la calle 2 marcadores de peso molecular y en las calles 3 a 5 muestras de EPO obtenidas según el método reivindicado.

La Fig. 3 ilustra un análisis SDS-PAGE de una muestra de EPO pura obtenida según el método descrito, tratada con glicanasas. Los marcadores de peso molecular se

corrieron en las calles 1, 4 y 8. En las calles 2 y 7 se ve EPO sin tratar. En la calle 3 se corrió EPO tratada con O-glicanasa, se verifica la presencia de una O-glicosilación. En la calle 5 se corrió EPO parcialmente degradada con N-glicanasa, se verifica la presencia de 3 N-glicosilaciones con los pesos moleculares correspondientes a los esperados para la EPO. En la calle 6 se corrió EPO degradada con O-glicanasa y N-glicanasa, obteniéndose el peso molecular esperado para la proteína totalmente deglicosilada.

La Fig. 4 ilustra un estudio de los puntos isoeléctricos de muestras de EPO pura producidas según el método descrito. Las muestras de EPO se corrieron en las calles 2, 3 y 4, los marcaddores de punto isoeléctrico en las calles 1 y 5. Se verifica la presencia de las formas correspondientes a EPO, con puntos isoeléctricos comprendidos entre 3,0 y 4,5.

VI. Reivindicaciones

Habiendo descrito y ejemplificado la naturaleza y objeto principal de la presente invención, como así también la manera en que la misma se puede llevar a la práctica, se declara reivindicar como de propiedad y de derechos exclusivos:

1. UN PROCEDIMIENTO DE CULTIVO MASIVO DE CÉLULAS RECOMBINANTES DE MAMÍFERO PARA LA OBTENCIÓN DE ERITROPOYETINA HUMANA RECOMBINANTE, caracterizado porque el medio de cultivo productivo contiene insulina como único agregado proteico.
2. UN PROCEDIMIENTO, según la reivindicación 1, caracterizado porque las células utilizadas son células CHO, COS, BHK, Namalwa, HeLa, Hep3B, HepG2 u otras células de mamífero.
3. UN PROCEDIMIENTO, según la reivindicación 1, caracterizado porque las células utilizadas son células CHO y COS.
4. UN PROCEDIMIENTO, según la reivindicación 1, caracterizado porque las células utilizadas son células CHO.
5. UN PROCEDIMIENTO, según la reivindicación 1, caracterizado porque la cantidad de insulina agregada es mayor que 1 mg/litro de medio de cultivo.
6. UN PROCEDIMIENTO, según la reivindicación 1, caracterizado porque la cantidad de insulina agregada es menor de 20 mg/litro de medio de cultivo.
7. UN PROCEDIMIENTO, según la reivindicación 1, caracterizado porque en las etapas de producción y cosecha las células se incuban a 37 °C y a 1 atmósfera de presión de aire de composición natural.

8. UN PROCEDIMIENTO, según la reivindicación 1, caracterizado porque utiliza un medio de cultivo libre de suero fetal bovino.

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9. UN PROCEDIMIENTO, según la reivindicación 1, caracterizado porque comprende:

   a) separar el sobrenadante de cultivo conteniendo EPO e insulina de las células productoras de EPO;

   b) concentrar el sobrenadante de cultivo de la etapa a);

   c) congelar el producto concentrado de la etapa b).

10. UN PROCEDIMIENTO, según la reivindicación 9, caracterizado porque el sobrenadante de la etapa b) es concentrado entre 50 y 150 veces.

11. UN PROCEDIMIENTO, según la reivindicación 9, caracterizado porque el sobrenadante de la etapa b) es concentrado 100 veces.

12. UN PROCEDIMIENTO, según la reivindicación 9, caracterizado porque la concentración de la etapa b) comprende el uso de un sistema de filtración tangencial com membranas porosas con un corte de peso molecular de 3.000 daltons.

13. UN PROCEDIMIENTO, según la reivindicación 9, caracterizado porque el producto concentrado de la etapa c) es filtrado a través de membranas con una porosidad de 0,22 µm de diámetro.

14. ERITROPOYETINA obtenida según el procedimiento caracterizado en la reivindicación 1.

BIO SIDUS S.A.
HUMBERTO M. DE PASQUALE
APODERADO

VII. Diagramas

Fig. 1. Análisis por Electroforesis en Gel de Poliacrilamida (SDS-PAGE)

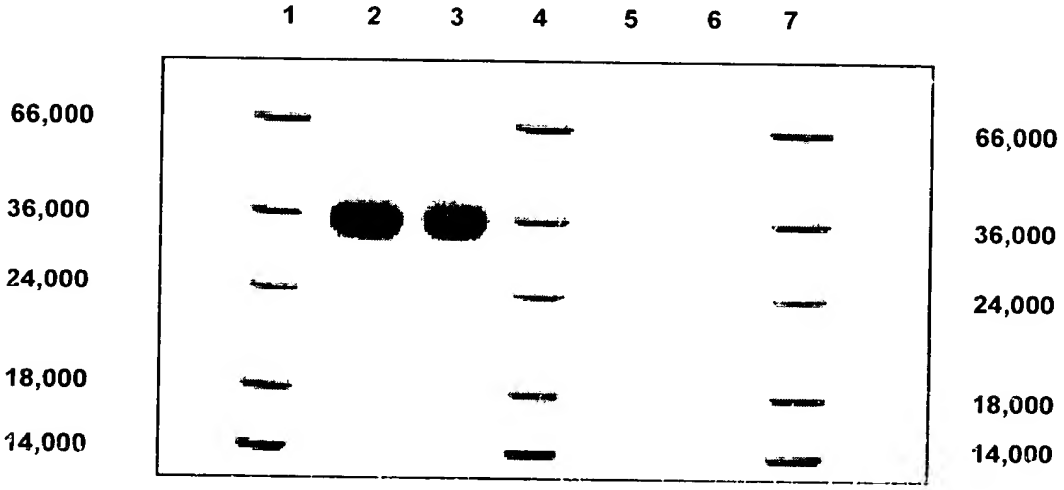



Fig. 2. Análisis "Western Blot"

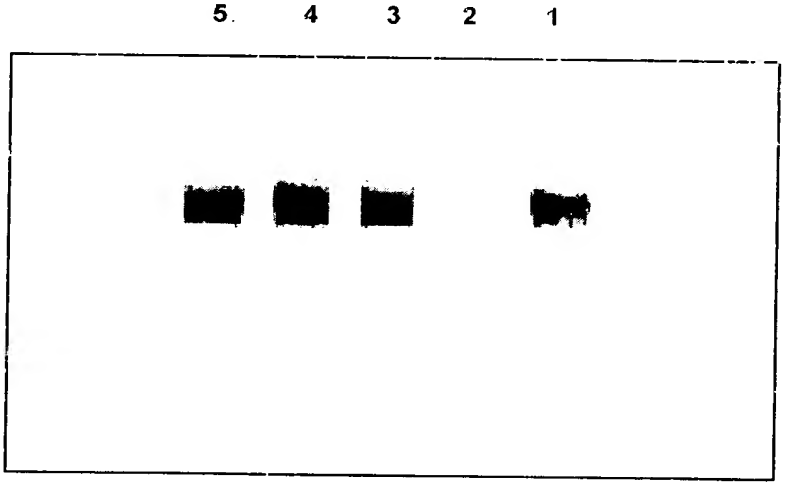


Fig. 3. Análisis por SDS-PAGE de la Digestión de EPO con Glicanasas

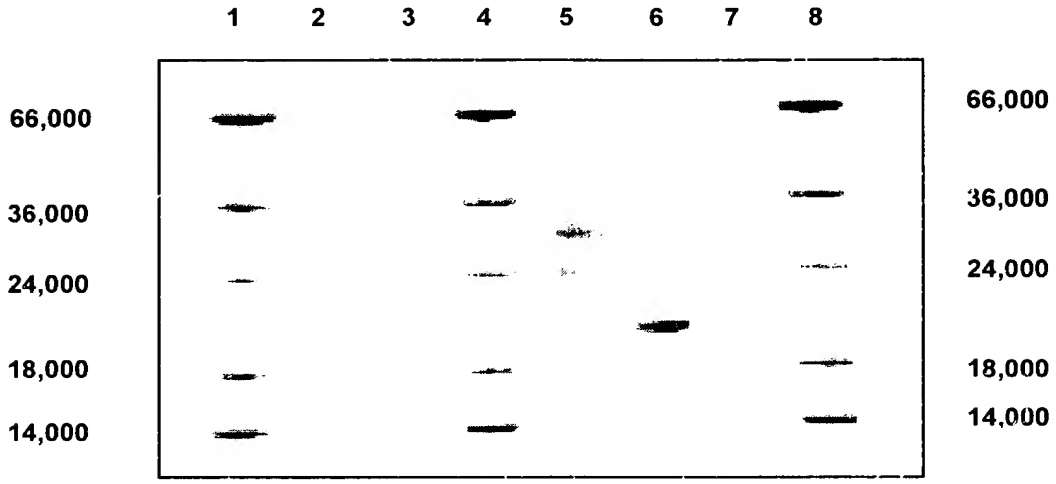
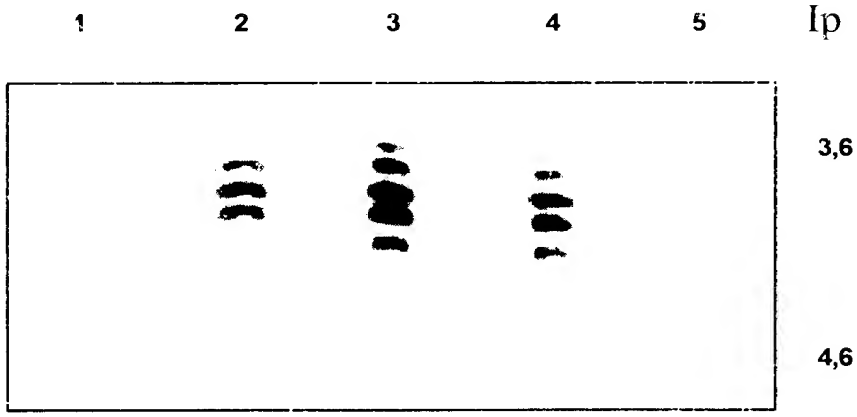



Fig. 4. Determinación de Punto Isoeléctrico (Isoelectroenfoque)



VIII. Resumen

La presente patente de invención describe un proceso de cultivo masivo de células de mamífero recombinantes, productoras de EPO. El proceso productivo sigue diferentes etapas de expansión, partiendo de células viables conservadas congeladas. Posteriormenente, se pasa a una etapa productiva en la que se utilizan medios de cultivo especialmente formulados para minimizar el agregado de suplementos proteicos.

La suplementación con insulina causa inesperdamente una elevada productividad de EPO que se encuentra con alta pureza en el medio de cultivo cosechado. Este es uno de los aspectos clave del método utilizado.

El sobrenadante de cultivo es finalmente concentrado para obtener mayor concentración de EPO en una forma apropiada para ser utilizada tal cual se obtiene o purificada ulteriormente para los usos que así lo requieran.

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IX. Referencias Adicionales


Adamson, "Epoetin Alfa: Into the New Millenium", Semin. Onc., 3 (7): 76-79 (June 25, 1998)

Alt et al., "Selective Multiplication of Dihydrofolate Reductase Genes in Methotrexate-resistant Variants of Cultured Murine Cells", J. Biol. Chem., 253: 1357-1370 (1978)

Andersen, et al., "The Effect of Cell-Culture Condictions in Oligosaccharide Structures of Secreted Glycoproteins", Curr. Op. Biotech., 5:546-549 (1994);

Anderson et al.,"Erythropoietin for the Treatment of Porphyria Cutanea Tarda in a Patient on Long-Term Hemodialysis" N. England J. of Med., 322 (5): 315-317 (1990)

Annable et al., "The Second International Reference Preparation of Erythropoietin, Human, Urinary, for Bioassay", Bull Wld. Hlth. Org., 47: 99-112 (1972)

Baciu et al., "Erythropoietin Interaction with the Mature Red Cell Membrane", Ann. N.Y. Acad. Sci., 414, 66-72 (1983)

Banerji et al., "Expression of a -Globin Gene is Enhanced by Remote SV40 DNA Sequences Cell", (part i) 27: 299-308 (1981)

Barthomeuf et al., "L'EPO Recombinante", Biofutur, 155: 16 (1996)

Battersby et al., "Isoforms of Recombinant Human Erythropoietin", Pathophysiology and Pharmacology of Erythropoietin. Springer-Verlag (1992)

Begin, "Prediction Response to Treatment with Recombinant Human Erythropoetin in Anaemia Associated with Cancer", Med. Oncol., 15 (Suppl. 1): 38-46 (1998)

Benoist et al., "In Vivo Sequence Requirements of the SV40 Early Promoter Region", Nature, 290: 304-310 (1981)

Benton et al., "Screening .lambda.gt Recombinant Clones by Hybridization to Single Plaques in Situ", Science, 196, 180-182 (Apr. 8. 1977)

Benz et al., "Hemoglobin Switching in Sheep", J. Biol. Chem., 5025-5032 (1978)

Berk et al., "Sizing and Mapping of Early Adenovirus mRNAs by Gel Electrophoresis of S1 Endonuclease-Digested Hybrids", Cell, 12: 721-732 (1977)

Billat et al., "In Vitro and In Vivo Regulation of Hepatic Erythropoiesis by Erythropoietin and Glucocorticoids in the Rat Fetus", Exp. Hematol., 10 (1), 133-140

(1982)

Bos et al., "Eukaryothic Expression of Cloned cDNA Coding for Influenza Viral Glycoproteins Using an SV40 Vector: Use of Recombinant DNA Mutants to Study Structure-Function Relationships.sup.1" Proc Symp. Mol. Biol. Negat., Strand Viruses Meeting, pp. 125-130, Compans et al., Eds.. Academic Press, San Diego, California (1984)

Bostock et al., "Gene Amplification in Methotrexate-resistant Mouse Cells", Mol. Biol., 153: 219-236 (1981)

Brandan, et al., "In Vitro Assay of Erythropoietin in Fetal Mouse Liver Cultures. 1. Comparison of Radioactive Tracers and Evidence of Assay Specificity", British J. Hematol., 47: 461-468 (1981)

Bray et al., "Human cDNA Clones for Four Species of G.alpha.-signal Transduction Protein", P.N.A.S. (USA), 83, 8893-8897 (Dec. 1986)

Breslow et al., "Isolation and Characterization of cDNA Clones for Human Apolipoprotein A-I", P.N.A.S. (USA), 79, 6861-6865 (Nov. 1982)

Brown et al., "Relationship of Amplified Dihydrofolate Reductase Genes to Double Minute Chromosomes in Unstably Resistant Mouse Fibroblast Cell Lines", Mol. Cell Biol., 1 (12): 1077-1083 (1981)

Browne et al., "Erythropoietin: Gene Cloning, Protein Structure, and Biological Properties", Cold Spring Harbor Symposia on Quantitative Biology, L1, 693-702 (1986)

Camiscoli et al., "Comparative Assay of Erythropoietin Standards", Ann. N.Y. Acad. Sci., 149: 40-45 (1968)

Canaani et al., "Regulated Expression of Human Interferon .beta.1 Gene after Transduction into Cultured Mouse and Rabbit Cells", P.N.A.S. (USA), 79, 5166-5170 (Sep. 1982)

Canadian Erythropoietin Study Group, "Association between Recombinant Human Erythropoietin and Quality of Life and Exercise Capacity of Patients Receiving Haemodialysis", BMJ, 300 (3): 573-578 (1990)

Casadevall, "Treatment of Anaemia with RHuEPO in Patients with MDS", Med. Oncol., 15 (Suppl. 1): 35-47 (1998)

Cazzola, "How and When to Use Erithropoietin", Curr. Op. Hetmatol., 5 (2): 103-108 (Mar. 1998)

Chan et al., "Construction and Selection of Recombinant Plasmids Containing Full-length Complementary DNAs Corresponding to Rat Insulins I and II", P.N.A.S. (USA), 76(10), 5036-5040 (Oct. 1979)

Chernajovsky et al., "Efficient Constitutive Production of Human Fibroblast Interferon by Hamster Cells Transformed with the IFN Gene Fused to an SV40 Early Promoter", DNA, 3: 297-308 (1982)

Chapman et al., "Amplification and Hormone-regulated Expression of a Mouse Mammary Tumor Virus-Eco gpt Fusion Plasmid in Mouse 3T6 Cells", Mol. Cell. Biol., 3: 1421-1429 (1983)

Chasin et al., "Mutant Alleles for Hypoxanthine Phosphoribosyltransferase: Codominant Expression, Complementation and Segregation in Hybrid Chinese Hamster Cells", Somatic Cell Genetics, 453-467 (1976)

Chen et al., "The Primary Structure and Genetic Organization of the Bovine Papillomavirus Type 1 Genome", Nature, 299: 529-534 (1982)

Chiba et al., "Stabilization of Urinary Erythropoietin", Biochem. Biophys. Res. Commun., 47(6), 1372-1377 (1972)

Choo et al., "Molecular Cloning of the Gene for Human Anti-haemophilic Factor IX", Nature, 299, 178-180 (Sep. 9, 1982)

Choppin et al., "Characterization of Erythropoientin Produced by IW32 Murine Erythroleukemia Cells", Blood, 64(2), 341-347 (Aug. 1984)

Christman et al., "Amplification of Expression of Hepatitis B Surface Antigen in 3T3 Cells Cotransfected with a Dominant-acting Gene and Cloned Viral DNA", P.N.A.S. 79, 1815-1819 (Mar. 1982)

Claus-Walker et al., "Spinal Cord Injury and Serum Erythropoietin", Arch. Phys. Med. Rehabil., 65, 370-374 (Jul. 1984)

Colby et al., "Immunological Differentiation Between E. Coli and CHO Cell-Derived Recombinant and Natural Human .beta.-Interferons.sup.1", J. Immunol., 133(6), 3091-3095 (1984)

Collen et al., "Biological Properties of Human Tissue-Type Plasminogen Activator Obtained by Expression of Recombinant DNA in Mammalian Cells", J. of Pharmacology and Exp. Therapeutics, 231(1), 146-152 (1984)

Colman, "Cells That Secrete Foreign Proteins", TIBS, 435-437 (Dec. 1982)

Congote et al., "Isolation of Two Biologically Active Peptides, Erythrotropin I and Erythrotropin II from Fetal Calf Intestine", Biochem. Biophys. Res. Comm., 115(2), 477-483 (Sep. 15, 1983)

Congote et al., "The Erythrotropins, New Erythroid Cell Stimulating Factors Extracted From Human and Bovine Fetal Tissues", Abstract 364, Proceedings 7th International Congress of Endocrinology, (Quebec City, Quebec, Jul. 1-7, 1984)

Congote, "High Performance Liquid Chromatographic Separation of Serum Erythrotropin and Erythropoietin", Chromatography, 310: 396-400 (1984)

Contrera et al., "Extraction of Erythropoietin from Kidneys of Hypoxic and Phenylhydrazine-treated Rats", Blood, 25(5), 809-816 (May 1965)

Corces et al., "Integration, Transcription and Control of a Drosophila Heat Shock Gene in Mouse Cells", Proc. Natl. Acad. Sci. (USA), 78(11): 7038-7042 (1981)

Corden et al., "Promoter Sequences of Eukaryotic Protein-coding Genes", Science, 209: 1406-1414 (1980)

Costantini et al., "Gene Transfer into the Mouse Germ-Line", J. Cell Physiol. Supp. 1, 219-226 (1982)

Cotes et al., "Changes in Serum Immunoreactive Erythropoietin during the Menstrual Cycle and Normal Pregnancy", Brit. J. Obstet. Gynaecol., 90, 304-311 (Apr. 1983)

Cotes et al., "Bio-Assay of Erythropoietin in Mice Made Polycythaemic by Exposure to Air at a Reduced Pressure", Nature, 191, 1065-1067 (Sep. 9, 1961)

Cotes, "Erythropoietin" Chapter: "Physiological Studies of Erythropoietin in Plasma", Jelkman and Gross Eds., 57-79 (1990)

Crouse et al., "Expression and Amplification of Engineered Mouse Dihydrofolate Reductase Minigenes", Mol. Cell. Biol., 3:257-266 (1983)

Craig Crowley et al., "Plasmid-directed Synthesis of Hepatitis B Surface Antigen in Monkey Cells", Mol. Cell. Biol., 3: 44-55 (1983)

Dainiak et al., "Mechanisms of Abnormal Erytropoiesis in Malignancy", Cancer, 51(6), 1101-1106 (1983)

Danko et al., "Epoetin Alfa for Treatment of Postpartum Anaemia", The Lancet, 334: 737-738 (1990)

Das et al., "Use of Synthetic Oligonucleotide Probes Complementary to Genes for

Human HLA-DR.alpha. and .beta. as Extension Primers for the Isolation of 5'-specific Genomic Clones", P.N.A.S. (USA) 80, 1531-1535 (Mar. 1983)

Davis et al. "A Manual for Genetic Engineering, Advanced Bacterial Genetics", Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 55-58, 174-176 (1983)

Davis et al., "Active Influenza Virus Neuraminidase is Expressed in Monkey Cells from cDNA Cloned in Simian Virus 40 Vectors", Proc. Natl. Acad. Sci. (USA), 80, 3976-3980 (1983)

Davis et al., "Characterization of Recombinant Human Erythropoietin Produced in Chinese Hamster Ovary Cells", Biochem 26: 2633-2638 (1987)

De Saint Vincent et al., "The Cloning and Reintroduction into Animal Cells of a Functional CAD Gene, a Dominant Amplifiable Genetic Marker ", Cell, 27: 267-277 (1981)

DeGowin et al., "The Mouse with Hypoxia-induced Erythremie, an Erythropoietin Bioassay Animal", J. Lab. Clin. Med. 60 (5): 846-852 (1962)

Derynck et al., "Human Transforming Growth Factor-.alpha.: Precursor Structure and Expression in E. Coli", Cell, 38, 287-297 (Aug. 1984)

Dessypris et al., "Effect of Pure Erythropoietin on DNA-synthesis by Human Marrow Day 15 Erythroid Burst Forming Units in Short-term Liquid Culture", Brit. J. Haematol., 56, 295-306 (1984)

Devos et al., "Purification of Recombinant Glycosylated Human Gamma Interferon Expressed in Transformed Chinese Hamster Ovary Cells", Interferon Research, 4, 461-468 (1984)

DiMaio et al., "Bovine Papillomavirus Vector that Propagates as a Plasmid in Both Mouse and Bacterial Cells", Proc. Natl. Acad. Sci., 79: 4030-4034 (1982)

DiMaio et al., "High-level Expression of a Cloned HLA Heavy Chain Gene Introduced Into Mouse Cells on a Bovine Papillomavirus Vector", Mol. Cell. Biol., 4: 340-350 (1984)

Dolnick et al., "Correlation of Dihydrofolate Reductase Elevation with Gene Amplification in a Homogeneously Staining Chromosomal Region in L5178Y Cells", J. Cell Biol., 83: 394-402 (1979)

Dordal et al., "Function and Composition of the Carbohydrate Portion of Human Urinary Erythropoietin", Experimental Hematology, 10, Supp. 11, p. 133, Abstract No. 222 (1982)

Dordal et al., "The Role of Carbohydrate in Erythropoietin Action", Endocrinology, 116(6), 2293-2299 (1985)

Draganac et al. "Rapid Preparation of Human Urinary Erythropoietin by High Performance Liquid Chromatography", Exptal. Hematol., 11(supl. 14): 58 (1983)

Dubé et al., "Glycosilation at Specific Sites of Erythropoietin is Essential for Biosynthesis, Secretion, and Biological Function", J. of Biol. Chem., 263 (33): 17516-16521 (1988)

Dubois et al., "The Development of Indications for the Preoperative Use Of Recombinant Erythropoietin", Canc J. Surg., 41 (5): 351-365 (1998)

Dukes et al., "Erythropoietin: a Complex with Different In Vivo and In Vitro Activities", J. Lab. & Clin. Med. 76(3): 439-444 (1970)

Dunn et al., "Use of a Computer Model in the Understanding of Erythropoietic Control Mechanisms", Chemical Abstracts, 91. 190417r (1979)

Dunn et al., "Current Concepts in Erythropoiesis". John Wiley & Sons. Chichester, England, (1983)

Dunn et al., "Serum Erythropoietin Titers during Prolonged Bedrest; Relevance to the Anaemia of Space Flight", Eur. J. Appln. Physiol., 52. 178-182 (1984)

Dunn et al., "Erythropoietin Bioassays Using Fetal Mouse Liver Cells: Validations and Technical Improvements", Exp. Hematol., 11(7), 590-600 (Aug. 1983)

Edman et al., "A Protein Sequentator", Eur. J. Biochem. 1, 80-91 (1967)

Elder et al., "Simian Virus 40 as an Eukaryotic Cloning Vehicle", Ann. Rev. Genet., 15: 295-340 (1981)

Edmunds et al., "Blood Pressure and Erythropoietin", The Lancet, 351-2 (February 13, 1988)

Emmanouel et al., "Metabolism of Pure Human Erythropoietin in the Rat", Am. J. Physiol., 247 (1 Pt 2), F168-76 (1984)

Ersley, "Erythropoietin Coming of Age", N. England J. of Med. 316 (2): 101-103 (1987)

Eschbach et al., "Correction by Erythropoietin (EPO) Therapy of the Anemia of Chronic Renal Failure (CRP) in Sheep", Clin. Res. 29(2), 518A (1981)

Eschbach et al., "The Anemia of Chronic Renal Failure in Sheep", J. Clin. Invest., 74(2), 434-441 (Aug. 1984)

Eschbach et al., "Correction of the Anemia of End-stage Renal Disease with Recombinant Human Erythropoietin", N. England J. of Med 316 (2):73-78 (1987)

Eschbach et al., "Recombinant Human Erythropoietin: Implications for Nephrology", Am. J. of Kidney Diseases XI (3): 203-209 (1988)

Eschbach, "The Anemia of Chronic Renal Failure: Pathophysiology and the Effects of Recombinant Erythropoietin", Kidney Int., 35: 134-148 (1989)

Espada et al., "A New Method for Concentration of Erythropoietin from Human Urine", Biochemical Medicine. 3: 475-484 (1970)

Espada et al., "Purificación de Eritropoyetina Urinaria Humana", Acta Physiol. Latinoamer., 20: 122-129 (1970)

Espada et al., "Purification of Human Urinary Erythropoietin", Fed. Proc., 41. 1159 (1982)

Farber et al., "Translation of mRNA from Human Kidneys into Biologically Active Erythropoietin Following Microinjection into Xenopus Laevis Oocytes", J. Lab. Clin. Med., 102, 681 abstract (Nov. 1983)

Farber et al., "Translation of mRNA from Anemic Baboon Kidney into Biologically Active Erythropoietin", Exp. Hematol., 11, Supp. 14, Abstract 101 (1983)

Farber, "Translation of RNA from Human Kidneys into Biologically Active Erythropoietin Following Microinjection into Xenopus Laevis Oocytes", Clin. Res., 31(4), 769A (Nov. 1983)

Farber et al., "Translation of mRNA from Human Kidneys into Biologically Active Erythropoietin Following Microinjection into Xenopus Laevis Oocytes", Blood, 62(5), Supp. No. 1, Abstract 392, 122a (1983)

Fauld et al., "Epoetin (Recombinant Human Erythropoietin)", Drugs, 38 (6): 864-899 (1989)

Fiers et al., "The Human Firbroblast and Human Immune Interferon Genes and Their Expression in Homologous and Heterologous Cells", Phil. Trans. R. Soc. Lond., B299, 29-38 (1982)

Finch, "Erythropoiesis, Erythropoietin, and Iron", Blood, 60(6), 1241-1246 (Dec. 1982)

Firkin, "Recombinant Human Erythropoietin Enters the Pharmacopeia", Aust. N.Z. J. Med. 19: 279-280 (1989)

Fisher et al., "Cooperative Erythropoietic Assay of Several Steroid Metabolites in Polycythemic Mice", Steroids, 30(6), 833-845 (Dec. 1977)

Fisher, "Erythropoietin: Pharmacology, Biogenesis and Control of Production", Pharmacological Review, 24(3), 459-508 (1972)

Fisher, "Control of Erythropoietin Production", Proc. Soc. Exp. Biol. & Med., 173. 289-305 (1983)

Fisher et al., "Effects of Testosterone, Cobalt & Hypoxia on Erythropoietin Production in the Isolated Perfused Dog Kidney", Ann. N.Y. Acad. Sci., 75-87 (1967)

Flaharty et al., "Epoetin: Human Recombinant Erythropoietin", Clin. Phar., 8: 769-782 (1989)

Flaharty et al., "Pharmacokinetics and Erythropoietic Response to Human Recombinant Erythropoietin in Healthy Men", Clin. Pharmacol. Ther.. 47 (5): 557-564 (1990)

Food and Drug Administration, Department of Health and Human Services, Office of Biologics Research and Review Center for Drugs and Biologics, "Points to Consider in the Production and Testing of New Drugs and Biologicals produced by Recombinant DNA Technology (April 10, 1985)

Fukuda et al., "Survival of Recombinant Erythropoietin in the Circulation: The Role of Carbohydrates", Blood, 73 (1) 84-89 (1989)

Garcia et al., "Radioimmunoassay of Erythropoietin: Circulating Levels in Normal and Polycythemic Human Beings", J. Lab. Clin. Med., 99, 624-635 (May 1982)

Garcia et al., "Radioimmunoassay of Erythropoietin", Blood Cells, 5, 405-419 (1979)

Garcia et al., "Immunological Neutralization of Various Erythropoietins", Proc. Soc. Exptl. Biol. Med., 112, 712-714 (1963)

Garcia, "The Radioimmunoassay of Human Plasma Erythropoietin", First International Conference on Hematopoiesis, Regulation of Erythropoiesis (Milan) 1972, 132-155

Gasser et al., "Expression of Abbreviated Mouse Dihydrofolate Reductase Genes in Cultured Hamster Cells", P.N.A.S. (USA), 79, 6522-6526 (Nov. 1982)

Gene Screen, New England Nuclear, Catalog No. NEF-972.

Gething et al., "Comparison of Different Eukaryotic Vectors for the Expression of Hemagglutinin Glycroprotein of Influenza Virus", Modern Approaches To Vaccines, pp. 263-268, Chanock et al., Eds. Cold Spring Harbor Lab (1984)

Gething et al., "Cell Surface Expression of Influenza Haemagglutinin from a Cloned DNA Copy of the RNA Gene", Nature, 293: 620-625 (1981)

Gething et al., "Construction of Influenza Haemagglutin Genes that Code for Intracellular and Secrete Forms of the Protein", Nature, 300,598-603 (Dec. 16, 1982)

Ghosh et al., "Identification of a Promoter Component Involved in Positioning the 5'termini of Simian Virus 40 Early mRNAs", Proc. Natl. Acad. Sci., 78: 100-104 (1981)

Gibson et al., "An Evaluation of Serum Erythropoietin Estimation By a Hemagglutination Inhibition Assay in the Differential Diagnosis of Polycythemia". Pathology, 16, 155-156 (Apr. 1984)

Gluzman, "SV40-Transformed Simian Cells Support the Replication of Early SV40 Mutants", Cell, 23, 175-182 (Jan. 1981)

Goeddel et al., "Synthesis of Human Fibroblast Interferon by E. Coli", Nucleic Acids Res., 8 (18), 4057-4074 (1980)

Goeddel et al., "Human Leukocyte Interferon Produced by E. Coli is Biologically Active". Nature, 287:411-416 (Oct. 2, 1980)

Goldberg et al., "Regulation of the Erythropoietin Gene: Evidence that the Oxygen Sensor is a Heme Protein", Science, 242: 1412-1415 (1988)

Goldberg et al., "The Regulated Expression of Erythropoietin by Two Human Hepatoma Cell Lines", Proc. Natl. Acad. Sci. (USA), 84: 7972-7976 (1987)

Goldwasser et al., "Erythropoietin: Assay and Study of Its Mode of Action", Meth. in Enzymol., 37, 109-121 (1975)

Goldwasser, "From Protein to Gene to Protein: The Molecular Biology of Erythropoietin", Am. J. of Kidney Diseases, 18(4) Supp. 1, 10-13 (Oct. 1991)

Goldwasser, "Biochemical Control of Erythroid Development", Current Topics in Developmental Biology, A. Monroy and A.A. Noscona, Eds., 173-211, Academic Press, New York, New York (1966)

Goldwasser et al., "The Molecular Weight of Sheep Plasma Erythropoietin", J. of Biol. Chem., 247(16), 5159-60 (Aug. 25, 1972)

Goldwasser et al., "Progress in the Purification of Erythropoietin", Ann. N.Y. Acad. Sci., 149:49-53 (1968)

Goldwasser et al., "Part II. Chemistry and Purification of Erythropoietin", Ann. N.Y. Acad. Sci., 149: 49-53 (1968)

Goldwasser et al., "On the Mechanism of Erythropoietin-induced Differentiation", J. of Biol. Chem., 249(13), 4202-4206 (Jul. 10, 1974)

Goldwasser et al., "Purification of Erythropoietin", P.N.A.S. (USA), 68(4), 697-698 (Apr. 1971)

Goldwasser et al., "On the Purification of Sheep Plasma Erythropoietin", Erythropoiesis, 43-49 (1962)

Goldwasser et al., "Further Purification of Sheep Plasma Erythropoietin", Bioch. Biophys. Acta, 64, 487-496 (1962)

Goldwasser, "Some Thoughts on the Nature of Erythropoietin-Responsive Cells", J. Cell Physiol., 110 (Supp. 1), 133-135 (1982)

Goldwasser et al., "An Assay for Erythropoietin In Vitro at the Milliunit Level", Endocrinology, 97(2), 315-323 (Aug. 1975)

Goldwasser et al., "Erythropoietin and the Differentiation of Red Blood Cells", Fed. Proc. 34, 2285-2292 (Dec. 1975)

Goochee et al., "Enviromental Effects on Protein Glycosylation", Biotechnology, 8, 421-427 (May 1990)

Goochee et al., "The Oligosaccharides of Glycoproteins: Bioprocess Factors Affecting Oligosaccharide Structure and their Effect on Glycoprotein Properties", Biotechnology, 9, 1347-1555 (Dec. 1991)

Goodman et al., "Cloning of Homone Genes from a Mixture of cDNA Molecules", Meth. in Enzymol., 68, 75-90 (1979)

Goodnough et al., "Increased Preoperative Collection of Autologous Blood with Recombinant Human Erythropoietin Therapy", N. England J. of Med., 321 (17) (1989)

Goodnough, "The Use of Erythropoietin in the Enhancement of Autologous Tranfusion Therapy", Curr. Opin. Hematol., 2 (3): 214-218 (1995)

Goeddel, "Human Leukocyte Interferon Produced by E. Coli is Biologically Active",

Nature, 287: 411-416 (1980)

Gordon et al., "A Plasma Extract with Erythropoietic Activity", Proc. Soc. Expt. Biol. Med., 86:255-258 (1954)

Gorman C, et al., "High Efficiency DNA-mediated Transformation of Primate Cells", Science, 221: 551-553 (1983)

Gorman et al., "Expression of Recombinant Plasmids in Mammalian Cells Is Enhanced by Sodium Butyrate", Nucl. Acid Res., 11: 7631-7648 (1983)

Goto et al., "Production of Recombinant Human Erythropoietin in Mammalian Cells: Host-Cell Dependency of the Biological Activity of the Cloned Glycoprotein", Bio/Tech. 6, 67-71 (Jan. 1988)

Gough et al., "Molecular Cloning of cDNA Encoding a Murine Haematopoietic Growth Regulator, Granulocyte-Macrophage Colony Stimulating Factor", Nature, 309, 763-767 (1984)

Gray et al., "Expression of Human Immune Interferon cDNA in E. Coli and Monkey Cells", Nature, 295, 503-508 (Feb. 11, 1982)

Green et al., "Conserved Primary Sequences of the DNA Terminal Proteins of Five Different Human Adenovirus Groups", Proc. Natl. Acad. Sci. (USA), 76(9): 4380-4384 (1979)

Grundmann et al., "Characterization of cDNA Coding for Human Factor XIIIa", P.N.A.S. (USA), 83, 8024-8028 (Nov. 1986)

Grunstein et al., "Colony Hybridization", Meth. in Enzym. 68, 379-389 (1979)

Grunstein et al., "Colony Hybridization: A Method for the Isolation of Cloned DNAs that Contain a Specific Gene", P.N.A.S. (USA), 72(10), 3961-3965 (Oct. 1975)

Gruss et al., "Expression of Simian Virus 40-rat Preproinsulin Recombinant in Monkey Kidney Cells Use of Preproinsulin RNA Processing Signals", Proc. Natl. Acad. Sci. (USA), 78: 133-137 (1981)

Gruss et al., "Simian Virus 40 Tandem Repeated Sequences as an Element of the Early romoter", Proc. Natl. Acad. Sci. (USA), 78: 943-947 (1981)

Gubler et al., "A Simple and Very Efficient Method for Generating cDNA Libraries", Gene, 25, 263-269 (1983)

Gurney et al., "Studies on Erythropoiesis. VI. Erythropoietin in Human Plasma", J. Lab.

& Clin. Med., 50(4): 534-542 (1957)

Haddy, "Erythropoietin is Sickle Cell Disease", Am. Jour. Ped. Hematol./Oncol., 4(2), 191-196 (Summer 1982)

Haga et al., "Plasma Erythropoietin Concentrations During the Early Anemia of Prematurity", Acta. Pediatr. Scand., 72, 827-831 (1983)

Hagiwara et al., "Erythropoietin Production in a Primary Culture of Human Renal Carcinoma Cells Maintained in Nude Mice", Blood, 63(4), 828-835 (Apr. 1984)

Hambley et al., "Erythropoietin: an Old Friend Revisited", BMJ, 300 : 621-622. (1990)

Hamer et al., "Expression of the Chromosomal Mouse .beta.sup.maj -globin Gene Cloned in SV40", Nature, 281, 35-40 (Sep. 6, 1979)

Hamer et al.,"SV40 Recombinants Carrying Rabbit -globin Gene Coding Sequences", Cell, 17: 725-735 (1979)

Hamer et al., "A Mouse Globin Gene Promoter is Functional in SV40", Cell, 21, 697-708 (Oct. 1980)

Hammond et al., "Production, Utilization and Excretion of Erythropoietin: I. Chronic Anemias. II. Aplastic Crisis. III. Erythropoietic Effects of Normal Plasma", Ann. N.Y. Acad. Sci., 149, 516-527 (1968)

Hammond et al., "Paraneoplastic Erythrocytosis and Ectopic Erythropoietins", Ann. N. Y. Acad. Sci., 230: 219-27 (1974)

Hanahan et al., "Plasmid Screening at High Colony Density", Gene, 10, 63-67 (1980)

Hartman et al., "Human Influenza Virus Hemagglutinin is Expressed in Monkey Cells Using Simian Virus 40 Vectors", Proc. Natl. Acad. Sci. (USA), 79, 233-237 (1982)

Hauser et al., "Inducibility of Human .beta -interferon in Mouse L-cell Clones", Nature, 297, 650-654 (Jun. 24, 1982)

Haynes et al., "Constitutive, Long-term Production of Human Interferons by Hamster Cells Containing Multiple Copies of a Cloned Interferon Gene", Nucleic Acids Research, 11(3), 587-607 (1983)

Haynes et al., "Production of a Glycosylated Human Protein by Recombinant DNA Technology", Proc. Takeda Sci. Found. Symp. Biosci., 111-29 (1983)

Hellmann et al.,"Familial Erythrocytosis with Over-production of Erythropoietin", Clin.

Lab. Haemat., 5, 335-342 (1983)

Hewick et al., "A Gas-Liquid Solid Phase Peptide and Protein Sequenator", J. Biol. Chem., 256, 7990-7997 (Aug. 1981)

Henry et al., "Clinical Use of Erythropoietin", Curr. Opin. Hematol., 2 (2): 118-124, (1995)

Higashi et al., "Structure and Expression of a Cloned cDNA for Mouse Interferon-.beta" J. Biol. Chem., 258(15):9522-9529 (1983)

Hokke et al., "Sialylated Carbohydrate Chains of Recombinant Human Glycoproteins Expressed in Chinese Hamster Ovary Cells Contain Traces of N-glycolyneuraminic Acid", FEBS Letters, 275, 9-14 (1990)

Horowitz et al., "Expression of Chimeric Genes in the Early Region of SV40", Mol. and Appl. Genet., 2: 147-159 (1983)

Howley et al., "Molecular Characterization of Papilloma Virus", Cold Spring Harbor Conf. Cell Proliferation, 7: 233-247genomes (1980)

Hsiung et al., "Efficient Production of Hepatitis B Surface Antigen Using Bovine Papilloma Virus-metallothionein Vector", Mol. and Appl. Genet., 2: 497-506 (1987)

Hu et al., "DNA Sequence Required for Initiation of Transcription in Vitro from the Major Late Promoter of Adenovirus 2", Proc. Natl. Acad. Sci. (USA), 78: 820-824 (1981)

Huang et al., "Identification of Human Erythropoietin Receptor", Am. Soci. of Biological Chemists, Am. Assoc. of Immunologists. Fed. Pract. (USA) 43(7) Abst. 2770, p. 1891 (1984)

Huang et al., "Characterization of Human Erythropoietin cDNA Clones", Am. Soc. of Biological Chemists, Am. Assoc. of Immunologists. Fed. Pract. (USA), 43(6) Abst. 1795, p. 1724

Imagawa et al., "Regulatory Elements of the Erythropoietin Gene", Blood, 77 (2) 278-285 (1991)

Imai et al., "Physicochemical and Biological Comparison of Recombinant Human Erythropoietin with Human Urinary Erythropoietin", J. Biochem, 107, 352-359 (1990)

Ismail et al., "An Opportunity to Intervene: Erythropoietin for the Treatment of Anaemia in Pre-dialysis Patients, Nephrol. Dial. Transplant., 13 (1): 14-17 (1998)

Itakura et al., "Synthesis and Use of Synthetic Oligonucleotides", Ann. Rev. Biochem., 53, 323-356 (1984)

Jacobs et al., "Isolation and Characterization of Genomic and cDNA Clones of Human Erythropoietin", Nature, 313, 806-809 (Feb. 28, 1985)

Jacobsen et al., "Relative Effectiveness of Phenylhydrazine Treatment and Hemorrhage in the Production of an Erythropoietic Factor", Blood, 11:937-945 (1956)

Jacobson et al., "Role of the Kidney in Erythropoiesis", Nature, 179:633-634 (Mar 23, 1957)

Jaye et al., "Isolation of Human Anti-haemophilic Factor IX cDNA Clone Using a Unique 52-Base Synthetic Oligonucleotide Probe Deduced from the Amino Acid Sequence of Bovine Factor IX", Nucleic Acids Res. 11(8), 2325-2335 (1983)

Jelkman et al., "Extraction of Erythropoietin from Isolated Renal Glomeruli of Hypoxic Rats", Exp. Hematol., 11(7), 581-588 (Aug. 1983)

Johnston et al., "Rapid Spontaneous Dihydrofolate Reductase Gene Amplification Shown by Fluorescence-activated Cell Sorting", Proc. Natl. Acad. Sci. (USA), 80: 3711-3715 (1983)

Kajimura et al., "Cloning the Heavy Chain of Human HLA-DR Antigen Using Synthetic Oligodeoxyribonucleotides as Hybridization Probes", DNA, 2(3), 175-182 (1983)

Karin et al., "Expression and Regulation of a Human Metallothionein Gene Carried on an Autonomously Replicating Shuttle Vector", Proc. Natl. Acad. Sci. (USA), 80: 4040-4044 (1983)

Karn et al., "Novel Bacteriophage .lambda. Cloning Vector", P.N.A.S. (USA), 77, 5172-5176 (Sep. 1980)

Katsuoka et al., "Erythropoietin Production in Human renal Carcinoma Cells Passaged in Nude Mice and in Tissue Culture", Gann, 74, 534-541 (Aug. 1983)

Katz et al , "Studies on the Site of Production of Erythropoietin", Ann. N.Y. Acad. Sci., 149: 120-127 (1968)

Kaufman et al., "Amplified Dihydrofolate Reductase Genes in Instable Methotrexate-resistant Cells are Associated with Double Minute Chromosome", Proc. Natl. Acad. Sci. (USA), 76: 5669-5673 (1979)

Kaufman et al., "Construction of a Modular Dihydrofolate Reductase cDNA Gene:

Analysis of Signals Utilized for Efficient Expression", Mol. Cell. Biol., 2: 1304-1319 (1982)

Kaufman et al., "Amplification and Expression of Sequences Cotransfected with a Modular Dihydrofolate Reductase Complementary DNA Gene", J. Mol. Biol., 159, 601-621 (1982)

Kaufman et al., "Expression and Amplification of DNA Introduced into Mammalian Cells". Gene Amplification, pp. 245-250, RT Schimke, Cold Spring Harbor, New York, New York (1982)

Kaufman et al., "Evolution of Chromosomal Regions Containing Transfected and Amplified Dihydrofolate Reductase Sequences", Mol. Cell. Biol., 3: 699-711 (1983)

Kaufman R et al., "Coamplification and Coexpression of Human Tissue-Type Plasminogen Activator and Murine Dihydrofolate Reductase Sequences in Chinese Hamster Ovary Cells", Mol. and Cell. Biol., 5 (7) 1750-9 (1985)

Kawai et al., "New Procedure for DNA Transfection with Polycation and Dimethyl Sulfoxide", Mol. Cell. Biol., 4(6): 1172-1174 (1984)

Kennell, "Principles and Practices of Nucleic Acid Hybridization", Prog. Nucl. Acid Res. Mol. Biol., 11, 259-301 (1971)

Kieny et al., "Expression of Rabies Virus Glycoprotein from a Recombinant Vaccinia Virus", Nature, 312, 163-166 (1984)

Kingston et al., "Regulation of Transcription of the Adenovirus EII Promoter by EIa Gene Products: Absence of Sequence Specificity", Mol. Cell. Biol., 4(10): 1970-1977 (1984)

Koeller, "Clinical Guidelines for the Treatment of Cancer Related Anemia", Pharmacotherapy, 18 (1): 156-169 (1998)

Konrad, "Applications of Genetic Engineering to the Pharmaceutical Industry". Ann. N.Y. Acad. Sci., 413, 12-22 (1983)

Konwalinka et al., "A Miniaturized Agar Culture System for Cloning Human Erythropoietic Progenitor Cells" , Exp. Hematol., 12, 75-79 (1984)

Kornblihtt et al., "Isolation and Characterization of cDNA Clones for Human and Bovine Fibronectins", P.N.A.S. (USA), 80, 3218-3222 (June 1983)

Krane, "The Role of Erythropoietin in the Anemia of Chronic Renal Failure", Henry Ford Hosp. Med. J., 31(3), 177-181 (1983)

Krantz, "Erythropoietin", Blood, 77 (3): 419-434

Krystal, "A Simple Microassay for Erythropoietin Based on .sup.3 H-Thymidine Incorporation into Spleen Cells from Phenylhydrazine Treated Mice", Exp. Hematol.. 11(7), 649-660 (Aug. 1983)

Krystal et al., "CM Affi-gel Blue Chromatography of Human Urine: A Simple One-step Procedure for Obtaining Erythropoietin Suitable for In Vitro Erythropoietic Progenitor Assays", British J. Haematol., 58: 533-546 (1984)

Kuhn et al., "Gene Transfer, Expression, and Molecular Cloning of the Human Transferrin Receptor Gene", Cell, 37, 95-103 (1984)

Kurachi et al., "Isolation and Characterization of a cDNA Coding for Human Factor IX", P.N.A.S. (USA), 79, 6461-6464 (Nov. 1982)

Kuratowska et al., "Studies on the Production of Erythropoietin by Isolated Perfused Organs", Blood, 18:527-534 (1961)

Kurtz, "A New Candidate for the Regulation of Erythropoiesis: Insulin-like Growth Factor I", FEBS Letters, 149(1). 105-108 (Nov. 1982)

Kurtz, "Hormonal Inducibility of Rat 2u Globulin Genes in Transfected Mouse Cells". Nature, 291: 629-631 (1981)

Lafferty et al., "Ultrastructural, Immunocytochemical Localization of Presumptive Erythropoietin Binding Sites on Developing Erythrocytic Cells of Normal Human Bone Marrow", J. Hystochem. Cytochem., 29(1): 49-56 (1981)

Lai et al., "Ovalbumin is Synthesized in Mouse Cells Transformed with the Natural Chicken Ovalbumin Gene", P.N.A.S. (USA), 77(1), 244-248 (Jan. 1980)

Lai et al., "Structural Characterization of Human Erythropoietin", J. of Biol. Chem., 261, 3116-3121 (Mar. 5, 1986)

Lai, "Technical Improvements in Protein Microsequencing", Analytica Chimica Acta, 163, 243-248 (1984)

Lange et al., "Application of Erythropoietin Antisera to Studies of Erythropoiesis", Ann. N.Y. Acad. Sci., 149:281-291 (1968)

Lange et al., "Antisera to Erythropoietin: Partial Characterization of Two Different Antibodies", J. Lab. & Clin. Med., 73(1): 78-90 (1969)

Lappin et al., "The Effect of Erythropoietin and Other Factors on DNA Synthesis by Mouse Spleen Cells", Exp. Hematol., 11(7), 661-666 (Aug. 1983)

Lasky et al., "Production of an HSV Subunit Vaccine by Genetically Engineered Mammalian Cell Lines", Modern Approaches to Vaccines, pp. 189-194, Chanock et al., Eds. Cold Spring Harbor Lab. (1984)

Lathe, "Synthetic Oligonucleotide Probes Deduced from Amino Acid Sequence Data", J. Mol. Biol., 183, 1-12 (1985)

Laub et al., "Expression of the Human Insulin Gene and cDNA in a Heterologous Mammalian System", J. Biol. Chem., 258(10), 6043-6050 (May 25, 1983)

Laub et al., "Synthesis of Hepatitis B Surface Antigen in Mammalian Cells: Expression of the Entire Gene and the Coding Region", Viol., 48(1):271-280 (1983)

Lavi, "Carcinogen-mediated Amplification of Viral DNA Sequences in Simian Virus 40-Transformed Chinese Hamster Embryo Cells", Proc. Natl. Acad. Sci. (USA), 78: 6144-6148 (1981)

Law et al., "A Stable Bovine Papillomavirus Hybrid Plasmid that Expresses a Dominant Selective Trait", Mol. Cel. Biol., 3(11): 2110-2115 (1983)

Lee et al., "Glucocorticoids Regulate Expression of Dihydrofolate Reductase cDNA in Mouse Mammary Tumour Virus Chimeric Plasmids", Nature, 294: 228-232 (1981)

Lee-Huang, "The Erythropoietin Gene", Oncogenes, Genes and Growth Factors, Chap. 7, pp. 199-222, Gordon Garaff, Ed., John Wiley & Sons, Boston, Massachusetts (1987)

Lee-Huang, "Cloning of Human Erythropoietin", Biophysical J., 45(Part 2 of 2), ABT M-PM-A12, p. 30a (1984)

Lee-Huang, "Monoclonal Antibodies to Human Erythropoietin", Abstract No. 1463, Fed. Proc., 41, 520 (1982)

Lee-Huang, "A New Preparative Method for Isolation of Human Erythropoietin with Hydrophobic Interaction Chromatography", Blood, 56(4), 620-624 (Oct. 1980)

Lee-Huang, "Cloning and Expression of Human EPO cDNA in E. Coli", P.N.A.S.(USA), 81, 2708-2712 (May 1984)

Levine et al., "Perioperative Recombinant Human Erythropoietin", Surgery 106 (2): 432-438, (1989)

Lewin, Genes, p. 307., John Wiley & Sons, Boston, Massachusetts (1983)

Lewis et al., "Selective Amplification of Polymorphic Dihydrofolate Reductase Gene Loci in Chinese Hamster Lung Cells", Proc. Natl. Acad. Sci. (USA), 79: 6961-6965 (1982)

Lewis et al., "Gene Amplification Accompanies Low Level Increases in the Activity of Dihydrofolate Reductase in Antifolate-resistant Chinese Hamster Lung Cells Containing Abnormally Banding Chromosomes", J. Cell. Biol., 94: 418-424 (1982)

Lin et al., "Cloning and Expression of the Human Erythropoietin Gene", Proc. Natl. Acad. Sci. (USA), 82, 7580-7584 (Nov. 1985)

Lin et al., "Monkey Erythropoietin Gene: Cloning, Expression and Comparison with the Human Erythropoietin Gene", Gene, 44, 201-209 (1986)

Lin et al., "Cloning of the Monkey EPO Gene", Abstract. J. Cell. Bioch., Suppl. 8B, p. 45 (Mar. 31-Apr. 24, 1984)

Lin et al., "Cloning and Expression of Monkey and Human Erythropoietin", Exp. Hematol. 12, 357 (1984)

Linman et al., "Studies on the Erythropoietic Effects of Hyperbaric Hyperoxia", Ann. N.Y. Acad. Sci., 149: 25-33 (1968)

Lipschitz et al., "Effect of Age on Hematopoiesis in Man", Blood, 63(3), 502-509 (Mar. 1983)

Liu Ch et al., "Direct Expression of Hepatitis B Surface Antigen in Monkey Cells from an SV40 Vector", DNA, I: 213-221 (1982)

Lusky et al., "Inhibition of SV40 Replication in Simian Cells by Specific pBR DNA Sequences", Nature, 293: 79-81 (1981)

Maniatis et al., "The Isolation of Structural Genes from Libraries of Eucaryotic DNA", Cell, 15, 687-701 (Oct. 1978)

Maniatis et al., "Molecular Cloning, a Laboratory Manual", pp. 5, 197-199, 392-393, 479-487, 493-503 Cold Springs Harbor, N.Y. (1982)

Maxam et al., "Sequencing End Labeled DNA with Base-Specific Chemical Cleavages", Methods in Enzymol., 65, 499-560 (1980)

Macdougall et al., "Pharmacokinetics of Recombinant Human Erythropoietin in Patients on Continous Ambulatory Peritoneal Dialysis", The Lancet, 425-427 (February 25,1989)

Macdougall et al., "Treating Renal Anaemia with Recombinant Huamn Erythropoietin: Practical Guidelines and a Clinica Algorithm", Br. Med. J., 300 (10): 655-659 (1990)

Macdougall, "Meeting the Challenges of the New Millennium: Optimizing the Use of Recombinant Human Erythropoietin" Nephrol. Dial. Transplant., 13 (2):23-27 (1998)

MacMillan et al., "Recombinant Human Erythropoietin in Children with Cancer", J. Pediatr. Hematol. Oncol., 20 (3): 187-189 (1998)

Maroteaux et al., "Sequences Involved in the Regulated Expression of the Human Interferon- 1 Gene in Recombinant SV40 DNA Vectors Replicating in Monkey Cells", The EMBO J. 1983, 2(3): 325-332

McCormick et al., "Regulated Expression of Human Interferon Genes In Chinese Hamster Ovary Cells", DNA, 2(1): 86 Abst 86 (1983)

McCormick et al., "Inducible Expression of Amplified Human Beta Interferon Genes in CHO Cells", Mol. Cell. Biol., 4(1):166-172 (Jan. 1984)

McDonald et al., "Cloning, Sequency, and Evolutionary Analysis of the Mouse Erythropoietin Gene", Mol. Cell. Biol., 6(3): 842-848

McGonigle et al., "Erythropoietin Deficiency and Inhibition of Erythropoiesis in Renal Insufficiency", Kidney Intl., 25(2), 437-444 (1984)

Meier et al., "Alpha.sub.1 -and Beta.sub.2 -Adrenergic Receptors Co-Expressed on Cloned MDCK Cells are Distinct Glycoproteins", Biochem. & Biophys. Res. Comm., 118(1), 73-81 (1984)

Mellon et al., "Identification of DNA Sequences Required for Transcription of the Human .alpha.1-Globin Gene in a New SV40 Host-Vector System", Cell, 27, 279-288 (Dec. 1981)

Mellor et al., "Expression of Murine H-2K.sup.b Histocompatibility Antigen in Cells Transferred with Cloned H-2 Genes", Nature, 298:529-534 (Aug. 1982)

Messing, "New M13 Vectors for Cloning", Methods in Enzymology, 101, 20-78 (1983)

"Methods in Yeast Genetics", P. 62, Cold Spring Harbor Lab., Cold Spring Harbor, New York (1983)

Miller et al., "Plasma Levels of Immunoreactive Erythropoietin after Acute Blood Loss in Man", Brit. J. Haematol., 52, 545-549 (1982)

Mirand, "Extra-renal and Renal Control of Erythropoietin Production", Ann. N.Y. Acad. Sci., 149:94-106 (1968)

Mirand et al., "Current Studies on the Role of Erythropoietin on Erythropoiesis", Ann. N.Y. Acad. Sci., 77:677-702 (1959)

Mishina et al., "Expression of Functional Acetylcholine Receptor from Cloned cDNA". Nature, 307: 604-608 (1984)

Mitrani-Rosenbaum et al., "Inducible Expression of the Human Interferon 1 Gene Linked to a Bovine Papilloma Virus DNA a Vector and Maintained Extrachromosomally in Mouse Cells", Mol. Cell. Biol., 3: 233-240 (1983)

Miyake et al., "Purification of Human Erythropoietin", J. Biol. Chem., vol. 252(15), 5558-5564 (Aug. 1977)

Mladenovic et al., "Anemia of Chronic Renal Failure (CRF) in the Sheep: Response to Erythropoietin (EP) In Vivo and In Vitro", Blood, 58(5), Suppl. 1, 99a (1981)

Moia et al., "Improvement in the Haemostatic Defect of Uraemia after treatment with Recombinant Human Erythropoietin", The Lancet, 1227-1229 (November 28, 1987)

Moreau et al., "The SV40 72 Base Repair Repeat has a Striking Effect on Gene Expression both in SV40 and Other Chimeric Recombinants", Nucl. Acid Res., 9: 6047-6067 (1981)

Moriarty et al., "Expression of the Hepatitis B Virus Surface Antigen Gene in Cell Culture by Using a Simian Virus 40 Vector", P.N.A.S. (USA), 78(4):2606-10 (Apr. 1981)

Mujovic et al., "The Effect of Indomethacin on Erythropoietin Production in Dogs Following Renal Artery Constriction. The Possible Role of Prostaglandins in the Generation of Erythropoietin by the Kidney", J. Pharmacol. Exp. Ther., 191: 575-581 (1974)

Mulligan et al., "Factors Governing the Expression of a Bacterial Gene in Mammalian Cells", Mol. Cell. Biol., 1: 449-459 (1981)

Mulligan et al., "Synthesis of Rabbit -globin in Cultured Monkey Kidney Cells Following Infection with a SV40 -globin Recombinant Genome", Nature, 277: 108-114 (1979)

Mulligan et al., "Expression of a Bacterial Gene in Mammalian Cells", Science, 209: 1422-1427 (1980)

Mulligan et al., "Selection for Animal Cells that Express the Escherichia Coli Gene Coding for Xanthine-guanine Phosphoribosyltransferase", Proc. Natl. Acad. Sci. (USA), 78: 2072-2076 (1981)

Murphy et al., "The Role of Glycoprotein Hormones in the Regulation of Hematopoiesis"
Acta. Haematologica Japonica, 46(7), 1380-1396 (Dec. 1983)

Murray et al., "Construction and Use of a Dominant, Selectable Marker: a Harvey Sarcoma Virus-dihydrofolate Reductase Chimera", Mol. Cel. Biol., 3(1): 32-43 (1983)

Myers et al., "Construction and Analysis of Simian Virus 40 Origins Defective in Tumor Antigen Binding and DNA Replication", P.N.A.S. (USA), 77, 6491-6495 (Nov. 1980)

Naets, "The Role of the Kidney in Erythropoiesis", J. Clin. Invest., 39:102-110 (1960)

Nakao et al., "Erythropoiesis in Anephric or Kidney Transplated Patients", Israel J. Med. Sci., 7:986-989 (Jul.-Aug. 1971)

Nathan et al., "Erythropoietin and the Regulation of Erythropoiesis", N. England J. of Med., 308(9), 520-522 (Mar. 3, 1983)

Naughton et al., "Evidence for an Erythropoietin-Stimulating Factor in Patients with Renal and Hepatic Disease", Acta. Haemat., 69, 171-179 (1983)

Naughton et al., "Evidence for a Hepatic-Renal Antagoinism in the Production of Hepatic Erythropoietin", Ann. Clin. Lab. Sci., 13(5), 432-438 (1983)

Nayak et al.,"Characterization of Influenza Virus Glycoproteins Expressed from Cloned cDNAs in Prokaryotic and Eukaryotic Cells", Modern Approaches To Vaccines, pp. 165-172, Chanock et al., eds., Cold Spring Harbor Lab. (1984)

Nielsen et al., "Erythropoietin b-D-galactosidase. The Generation, Purification and Use of Fusion Protein", J. Immunol. Meth., 111. 1-9 (1988)

Nigg et al., "Immunofluorescent Localization of the Transforming Protein of Rous Sarcoma Virus with Antibodies against a Synthetic src Peptide", P.N.A.S. (USA), 79, 5322-5326 (Sep. 1982)

Nimtz et al., "Structures of Sialylated Oligosaccharides of Human Erythropoietin Expressed in Recombinant BHK-21 Cells", Eur. J. Biochem, 213, 39-56 (1993)

Nunberg et al., "Amplified Dihydrofolate Reductase Genes are Localized to a Homogeneously Staining Region of a Single Chromosome in a Methotrexate-resistant

Chinese Hamster Ovary Cell Line", Proc. Natl. Acad. Sci. (USA), 75: 5553-5556 (1978)

O'Hare et al., "Transformation of Mouse Fibroblast to Methotrexate Resistance by a Recombinant Plasmid Expressing a Prokaryotic Dihydrofolate", Natl. Acad. Sci. (USA), 78: 1527-1531 (1981)

Ogle et al., "Production of Erythropoietin In Vitro: A Review", In Vitro, 14(11), 945-949 (1978)

Okayama et al.,"A cDNA Cloning Vector that Permits Expression of cDNA Inserts in Mammalian Cells", Mol. Cell. Biol., 3: 280-289 (1983)

Ohno et al., "Inducer-responsive Expression of the Cloned Human Interferon 1 Gene Introduced into Cultured Mouse Cells", Nucl. Acid. Res., 10(3): 967-977 (1982)

Osterborg, "Recombinant Human Erythropoetin (rHuEPO) Therapy in Patients with Cancer Related Anaemia: What Have We Learned?", Med. Oncol., 15 (Suppl. 1): 47-49 (1998)

Pankratz et al., "A Simple 3-Step Procedure for Purifying Baboon Urinary Erythropoietin to Apparent Homogeneity", Exp. Hematol., 11, Supp. 14, Abst. 102 (1983)

Papayannopoulou et al., "On the In Vivo Action of Erythropoietin: A Quantitative Analysis", J. of Clin. Investigation, 51, 1179-1185 (1972)

Parekh et al., "N-Glycosylation and In Vitro Enzymatic Activity of Human Recombinant Tissue Plasminogen Activator Expressed in Chines Hamster Ovary Cells and a murine Cell Line", Biochemistry, 28, 7670-7679 (1989)

Parker et al., "Regulation of Simian Virus 40 Transcription: Sensitive Analysis of the RNA Species Present Early in Infections by Virus or Viral DNA", J. Virol., 31(2): 360-369 (1979)

Pavlakis et al., "Regulation of a Metallothionein-growth Hormone Hybrid Gene in Bovine Papilloma Virus", Proc. Natl. Acad. Sci. (USA), 80: 397-401 (1983)

Pavlovic-Kentera et al., "Effects of Prostaglandin Synthetase Inhibitors, Salt Overload and Renomedullary Dissection on the Hypoxia Stimulated Erythropoietin Production in Rats", Exp. Hematol., 8(Supp. 8), 283-291 (1980)

Pennathur-Das et al., "Evidence for the Presence of CFU-E with Increased In Vitro Sensitivity to Erythropoietin in Sickle Cell Anemia", Blood, 63(5), 1168-71 (May 1984)

Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, Boston,

Massachusetts (1984)

Pitha et al., "Induction of Human .beta.-interferon Synthesis with Poly (rI.cndot.rC) in Mouse Cells Transfected with Cloned cDNA Plasmids", P.N.A.S. (USA), 79, 4337-4341 (Jul. 1982)

Powell et al., "Human Erythropoietin Gene: High Level Expression in Stably Transfected Mammalian Cells and Chromosome Localization", Proc. Natl. Acad. Sci. (USA), 83, 6465-6469 (Sep. 1986)

Quelle et al., "High-level Expression and Purification of a Recombinant Human Erythropoietin Producing a Baculovirus Vector", Blood, 74: 652-657 (1989)

Quelle et al., "Phosphorylatable and Epitope-Tagged Human Erythropoietins: Utility and Purification of Native Baculovirus-Derived Forms", Protein Expression and Purification 3, 461-469 (1992)

Radtke et al., "Serum Erythropoietin Concentration in Chronic Renal Failure: Relationship to Degree of Anemia and Excretory Renal Function", Blood, 54 (4): 877-884 (1979)

Ramabhadran et al., "Synthesis and Glycosylation of the Common .alpha. Subunit of Human Glycoprotein Hormones in Mouse Cells", Proc. Natl. Acad. Sci. (USA), 81, 6701-6705 (1984)

Rambach et al., "Acid Hydrolysis of Erythropoietin", Proc. Soc. Exp. Biol., 99, 482-483 (1958)

Recny et al., "Structural Characterization on Natural Human Urinary and Recombinant DNA-derived Erythropoietin", Biol. Chem., 262 (35): 17156-17163 (1987)

Recormon® Products Monograph, Renal Anaemia, Boehringer Manheim GmbH

Reddy et al., "Nucleotide Sequence Analysis of the Proviral Genome of Avian Myelocytomatosis Virus (MC29)", Proc. Natl. Acad. Sci. (USA), 80: 2500-2504 (1983)

Reilly et al., "Use of Synthetic Oligonucleotides to Clone Genomic DNA: Isolation of a tRNA.sup.Phe Gene from Mouse", DNA, 1:192 (1982)

Reissmann et al., "Erythropoietin Formation in Isolated Kidneys and Liver", Erithropoiesis, 71-77 (1962)

Rigby, "Expression of Cloned Genes in Eukaryotic Cells Using Vector Systems Derived from Viral Replicons", Genetic Engineering, R. Williamson, Ed., 3:83-140, Academic Press, London, England (1982)

Rigby , "Review Article: Cloning Vectors Derived from Animal Viruses", J. Gen. Virol., 64: 255-266 (1983)

Riggs et al.,"Synthetic DNA and Medicine", Am. J. Hum. Genet., 31, 531-538 (1979)

Ringold et al., "Co-Expression and Amplification of Dihydrofolate Reductase cDNA and the Escherichia coli XGPRT Gene in Chinese Hamster Ovary Cells", J. Mol. & Appl., Genetics, 1(3), 165-175 (1981)

Roh et al., "Plasma Disappearance of I.sup.125 labeled Human Uninary Erythropoietin in Rabbits", Fed. Proc., 29(2), 782 Abst. 3030 (1970)

Rose et al., "Expression from Cloned cDNA of Cell-Surface Secreted Forms of the Glycoprotein of Vesicular Stomatitis Virus in Eucaryotic Cells", Cell, 30, 753-762 (1982)

Rosso et al., "Use of Erithropoietin in Oncology", Tumori., 83 (4 Suppl.2): 26-30, (1997)

Roth et al., "Influenza Virus Hemagglutinin Expression Is Polarized in Cells infected with Recombinant SV40 Viruses Carrying Cloned Hemagglutinin DNA", Cell, 33, 435-443 (1983)

Rothmann et al., "Erythropoietin-Dependent Erythrocytosis Associated with Hepatic Angiosarcoma", J. Surg. Oncol., 20, 105-108 (1982)

Saito et al., "Translation of Human Erythropoietin-mRNAs", Exp. Hematol., 11(14). 228 (1983)

Saito et al., "In Vitro Assay of Erythropoietin: Simple Determination in a Small Amount of Human Serum Samples", Jap. J. Med., 23(1), 16-21 (Feb. 1984)

Sakata et al., "Plasma Erythropoietin Assay by a Fetal Mouse Liver Cell Culture Method with Special Reference to Effective Elimination of Erythroid Colony Inhibitor(s) in Plasma", Exp. Hematol., 15: 226-233 (1987)

Sambrook et al., "Expression of Proteins on the Cell Surface Using Mammalian Vectors", Experimental Manipulation of Gene Expression, pp. 225-246, Academic Press, London, England (1983)

Sanders et al., "Amplification and Cloning of the Chinese Hamster Glutamine Synthetase Gene", The EMBO J., 3: 65-71 (1984)

Sanger et al., "DNA Sequencing with Chain-terminating Inhibitors", P.N.A.S. (USA).

74, 5463-5467 (Dec. 1977)

Sarver et al., "Bovine Papilloma Virus Deoxyribonucleic Acid: A Novel Eukaryotic Cloning Vector", Mol. Cell. Biol., 1: 486-496 (1981)

Sarver et al., "Transformation and Replication in Mouse Cells of a Bovine Papillomavirus-pML2 Plasmid Vector that Can Be Rescued in Bacteria". Proc. Natl. Acad. Sci. (USA), 79: 7147-7151 (1982)

Sasaki, "Isolation of Erythropoietin by Monoclonal Antibody", Biomed. Biochim. Acta., 42(11/12), S202-206 (1983)

Sasaki et al., "Carbohydrate Structure of Erythropoietin Expressed in Chinese Hamster Ovary Cells by a Human Erythropoietin cDNA", J. Biol. Chem., 262(25), 12059-12070 (Sep. 5, 1987)

Sasaki et al., "Site-specific Glycosilation of Human Recombinant Erythropoietin: Analysis of Glycopeptides of Peptides at Each Glycosilation Site by Fast Atom Bombardment Mass Spectrometry", Bioch.,27: 8618-8626 (1988)

Saveria Campo et al., "Transcriptional Control Signals in the Genome of Bovine Papillomavirus Type 1", Nature, 303: 77-80 (1983)

Scahill et al., "Expression and Characterization of the Product of a Human Immune Interferon cDNA Gene in Chinese Hamster Ovary Cells", Proc. Natl. Acad. Sci. (USA), 80, 4654-4658 (1983)

Schaffner W, "Direct Transfer of Cloned Genes from Bacteria to Mammalian Cells", Proc. Natl. Acad. Sci., 77: 2163-2167 (1980)

Schimke et al., "Chromosomal and Extrachromosomal Localization of Amplified Dihydrofolate Reductase Genes in Cultured Mammalian Cells", Cold Spring Harbor Symp. Quant. Biol., 45: 785-797 (1981)

Schimke, "Gene Amplification in Cultured Animal Cells", Cell, 37: 705-713 (1984)

Seeburg et al., "Synthesis of Growth Hormone by Bacteria", Nature, 276, 795-798 (Dec. 1978)

Shahidi, "Androgens and Erythropoiesis", N. England J. of Med., 289, 72-80 (Jul. 12, 1973)

Sherwood et al., "Erythropoietin Titers in Sickle Cell Disease and Chronic Renal Failure", Blood, 58: 49[a] (1981)

Sherwood et al., "Extraction of Erythropoietin from Normal Kidneys", Endocrinology, 103(3), 866-870 (1978)

Sherwood et al., "A Radioimmunoassay for Erythropoietin", Blood, 54(4), 885-893 (Oct. 1979)

Sherwood et al., "Erythropoietin Titers in Sickle Cell Disease and Chronic Renal Failure", Blood, 58 (1), Supp. 1, Abstract 105 (1981)

Shiramizu et al., "Human Renal Carcinoma Cells Secreting Erythropoietin In Vivo and In Vitro", Blood, 78(10), Supp. 1 (Nov. 15, 1991)

Shoemaker et al., "Murine Erythropoietin Gene: Cloning, Expression, and Human Gene Homology", Mol. Cell. Biol., 6 (3): 849-858

Schumperli et al., "Efficient Expression of Escherichia Coli Galactokinase Gene in Mammalian Cells", Proc. Natl. Acad. Sci. (USA), 79: 257-261 (1982)

Siddiqui, "NOTE: Expression of Hepatitis B Virus Surface Antigen Gene in Cultured Cells by Using Recombinant Plasmid Vectors", Mol. Cell. Biol., 3: 143-147 (1983)

Simonsen et al., "Isolation and Expression of an Altered Mouse Dihydrofolate Reductase cDNA", Proc. Natl. Acad. Sci. (USA), 50: 2495-2499 (1983)

Singer-Sam et al., "Isolation of a cDNA Clone for Human X-linked 3-phosphoglycerate Kinase by Use of a Mixture of Synthetic Oligodeoxyribonucleotides as a Detection Probe", P.N.A.S. (USA), 80, 802-806 (Feb. 1983)

Smith et al., "Construction and Characterization of an Infectious Vaccinia Virus Recombinant that Expresses the Influenza Hemagglutinin Gene and Induces Resistance to Influenza Virus Infection in Hamsters", Proc. Natl. Acad. Sci. (USA), 80, 7155-7159 (1983)

Smith et al., "Production of Human Beta Interferon in Insect Cells Infected with a Baculovirus Expression Vector", Mol. Cell. Biol., 3(12): 2156-2165 (1983)

Smith Dordal et al., "The Role of Carbohydrate in Erythropoietin Action", Endocrinology, 116 (6): 2293-2299 (1985)

Sood et al., "Isolation and Partial Nucleotide Sequence of a cDNA Clone for Human Histocompatibility Antigen HLA-B by Use of an Oligodeoxynucleotide Primer", Proc. Natl. Acad. Sci. (USA), 78: 616-620 (1981)

Southern et al., "Transformation of Mammalian Cells to Antibiotic Resistance with a Bacterial Gene Under Control of the SV40 Early Region Promoter", J. Mol. Appl.

Genet., 1(4), 327-341 (1982)

Southern, "Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis", J. Mol. Biol., 98, 503-517 (1975)

Spellman et al., "Carbohydrate Structure of Recombinant Soluble Human CD4 Expressed in Chinese Hamster Ovary Cell", Biochemistry, 30(9), 2395-2406 (1991)

Spellman et al., "Carbohydrate Structure of Human Tissue Plasminogen Activator Expressed in Chinese Hamster Ovary Cells", J. of Biol. Chem., 264(24), 14100-14111 (Aug. 26, 1989)

Spivak, "Erythropoietin: A Brief Review", Nephron, 52: 289-294 (1989)

Spivak et al., "The In Vivo Metabolism of Recombinant Human Erythropoietin in the Rat", Blood, 73 (1): 90-99 (1989)

Stark et al., "Gene Amplification", Ann. Rev. Biochem., 53: 447-91 (1984)

Steinberg et al., "Erythropoietin Kinetics in Rats: Generation and Clearance", Blood,67 (3): 646-649 (1986)

Stenlund et al., "Secretion of Hepatitis B Virus Surface Antigen from Mouse Cells Using an Extra-chromosomal Eucaryotic Vector", The EMBO J., 1983, 2(5): 669-673

Stephenson et al., "Quantitative Assay Method for Erythropoietin In Vitro", Endocrinology, 88: 1519-1520 (1971)

Storring et al., "The International Standard for Recombinant DNA Derived Erythropoietin: Collaborative Study of Four Recombinant DNA Derived Erythropoietins and Two Highly Purified Human Urinary Erythropoietins", J. of Endo., 134, 459-84 (1992)

Stratowa et al., "Recombinant Retroviral DNA Yielding High Expression of Hepatitis B Surface Antigen", The EMBO J., 1(12): 1573-1578 (1982)

Subramani et al., "Expression of the Mouse Dihydrofolate Reductase Complementary Deoxyribonucleic Acid in Simian Virus SV 40 Vectors", Mol. Cell. Biol., 1: 854-864 (1981)

Sue et al., "Site-specific Antibodies to Human Erythropoietin Directed toward the NH.sub.2 -terminal Region", Proc. Nat. Acad. Sci. (USA), 80, 3651-3655 (1983)

Suggs et al., "Use of Synthetic Oligodeoxyribonucleotide for the Isolation of Specific Cloned DNA Sequences", Developmental Biology Using Purified Genes, 683-693, D.

Brown, Ed. (1981)

Suggs et al.,"Use of Synthetic Oligonucleotides as Hybridization Probes: Isolation of Cloned cDNA Sequences for Human B.sub.2 -microglobulin", P.N.A.S. (USA), 78, 6613-6617 (1981)

Sveda et al., "Functional Expression in Primate Cells of Cloned DNA Coding for the Hemagglutinin Surface Glycoprotein of Influenza Virus", Pros. Natl. Acad. Sci. (USA), 78(10):5488-5492 (Sep. 1981)

Sytowski et al., "The Biochemistry of Erythropoietin: An Approach to its Mode of Action", Exp. Hematol., 8(Supp. 8), 52-63 (1980)

Sytowski et al., "A Novel Radioimmunoassay for Human Erythropoietin Using a Synthetic NH.sub.2 -Terminal Polypeptide and Anti-Peptide Antibodies", J. Immunol. Methods, 69, 181-186 (1984)

Szostak et al., "Hybridization with Synthetic Oligonucleotides", Meth. in Enzymol , 68, 419-428 (1979)

Takeuchi et al., "Relationship between Sugar Chain Structure and Biological Activity of Recombinant Human Erythropoietin Produced in Chinese Hamster Ovary Cells", Proc. Natl. Acad. Sci. (USA), 86, 7819-7822 (Oct. 1989)

Takeuchi, "Comparative Study of the Asparagine-linked Sugar Chains of Human Erythropoietin Purified from Urine and the Culture Medium of Recombinant Chinese Hamster Ovary Cells", J. Biol. Chem., 263(8), 3657-3663 (Mar. 15, 1988)

Tambourin et al., "Production of Erythropoietin-like Activity by a Murine Erythroleukemia Cell Line", P.N.A.S. (USA), 80, 6269-6273 (1983)

Taub et al., "An Improved Method for Preparing Large Arrays of Bacterial Colonies Containing Plasmids for Hybridization: In Situ Purification and Stable Binding of DNA on Paper Filters", Chemical Abstracts, 97(23), 164, Abstract No. 194002y (Dec. 12, 1982)

Taub et al., "An Improved Method for Preparing Large Arrays of Bacterial Colonies Containing Plasmids for Hybridization: In Situ Purification and Stable Binding of DNA on Paper Filters", Anal. Biochem., 126, 222-230 (1982)

Testa et al., "Role of Purified Erythropoietin in the Amplification of the Erythroid Compartment", Exp. Hematol., 8(Supp. 8), 144-152 (1980)

Tong et al., "The Formation of Erythrocyte Membrane Proteins during Erythropoietin-induced Differentiation", J. Biol. Chem., 256(24), 12666-12672 (Dec. 25, 1981)

Toole et al., "Molecular Cloning of a cDNA Encoding Human Antihaemophilic Factor", Nature, 312, 342-347 (Nov. 8, 1984)

Tsuda et al., "Comparative Structural Study of N-Linked Oligosaccharides of Urinary and Recombinant Erythropoietins", Biochemistry, 27(15), 5646-5654 (1988)

Tsuda et al., " The Role of Carbohydrate in Recombinant Human Erythropoietin", Eur. J. Biochem., 188: 405-411 (1990)

Thummel et al., "Construction of Adenovirus Expression Vectors by Site-directed In Vivo Recombination". J. Mol. Appl. Genet., 1:435-446 (1982)

Thummel et al., "Translational Control of SV40 T Antigen Expressed from the Adenovirus Late Promoter", Cell, 33: 455-464 (1983)

Thurmon et al., "Hemoglobin Switching in Nonanemic Sheep. III. Evidence for Presumptive Identity between the A C Factor and Erythropoietin", Blood, 36(5): 598-606 (1970)

Udupa et al., "Erythropoiesis in the Aged Mouse", Lab. Clin. Med., 103(4), 574-580; 581-588 (1984)

Umemura et al., "The Mechanism of Expansion of Late Erythroid Progenitors During Erythroid Regeneration: Target Cells and Effects of Erythropoietin and Interleukin-3", Blood, 73 (7) 1993-1998 (1989)

Urlaub et al., "Isolation of Chinese Hamster Cell Mutants Deficient in Dihydrofolate Reductase Activity", Proc. Nat. Acad. Sci. (USA), vol. 77(7), 4216-4220 (Jul. 1980)

Van Stone et al., "Effect of Erythropoietin on Anemia of Peritoneally Dialyzed Anephric Rats", Kidney Intl., 15. 370-375 (1979)

Vedovato et al., "Erythropoietin Levels in Heterozygous Beta-Thalassemia", Acta. Haematol., 71, 211-213 (1984)

Viera et al., "The pUC Plasmids, an M13mp7-derived System for Insertion Mutagenesis and Sequencing with Synthetic Universal Primers", Gene, 19, 259-268 (1982)

Wahl et al., "Effect of Chromosomal Position on Amplification of Transfected Genes in Animal Cells", Nature, 307: 516-520 (1984)

Wasley et al., "The Importance of N-and O-Linked Oligosaccharides for the Biosynthesis and In Vitro and In Vivo Biologic Activities of Erythropoietin", Blood, 77 (12): 2624-2632 (1991)

Walker et al., Techniques in Molecular Biology, p. 280, Macmillan Pub. Co., New York, New York (1983)

Wallace et al., "The Use of Synthetic Oligonucleotides as Hybridization Probes. II. Hybridization of Oligonucleotides of Mixed Sequence to Rabbit .beta.-globin DNA", Nuc. Acids Res., 9(4), 879-894 (1981)

Wang et al., "Enhanced Production of Hepatitis B Surface Antigen in NIH 3T3 Fibroblast by Using Extrachromosomally Replicating Bovine Papillomavirus Vector", Mol. Cell. Biol., 3: 1032-1039 (1983)

Wang et al., "Some Chemical Properties of Human Erythropoietin", Endocrinology, 116(6), 2286-2292 (1985)

Wang et al., "Renal and Extrarenal Erythropoietin Production in Male and Female Rats of Various Ages", J. Lab. Clin. Med., 79(2), 181-186 (Feb. 1972)

Watson et al., "Structure Determination of the Intact Major Sialylated Oligosaccharide Chains of Recombinant Human Erythropoietin Expressed in Chinese Hamster Ovary Cells", Glycobiology, 4 (2) 227-237 (1994)

Weiland et al., "In Vivo Activity of Asialo-Erythropoietin in Combination with Asialo-Glycoproteins", Blut, 44(3), 173-175 (1982)

Weiss et al., "Characterization of a Monoclonal Antibody to Human Erythropoietin", P.N.A.S. (USA), 79, 5465-5469 (1982)

Weiss et al., "Studies of the Pathogenesis of Anemia of Inflammation: Mechanism of Impaired Erythropoiesis", Am. J. Vet. Res., 44(10), 1832-1835 (Oct. 1983)

Weissman et al., "Structure and Expression of Human IFN-.alpha. Genes", Phil. Trans. R. Soc. Lond., B299, 7-28 (1982)

White et al., "Studies on Erythropoietin", Recent Progr. Hormone Res., 16:219-262 (1960)

Wickens et al., "Expression of a Chicken Chromosomal Ovalbumin Gene Injected into Frog Oocyte Nucleo", Nature, 285:628-634 (26 Jun. 1980)

Wide et al., "Molecular Charge Heterogeneity of Human Serum Erythropoietin", British J. Haemat., 76, 121-127 (1990)

Wigler et al., "Transformation of Mammalian Cells with Genes from Procaryotes and Eucaryotes", Cell, 16: 777-785 (1979)

Wigler et al., "Biochemical Transfer of Single-copy Eucaryotic Genes Using Total Cellular DNA as Donor", Cell, 14: 725-731 (1978)

Wigler et al., "Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells", Cell, 11: 223-232 (1977)

Wigler et al., "Transformation of Mammalian Cells with an Amplifiable Dominant-Acting Gene", Proc. Natl. Acad. Sci. (USA), 77: 3567-3570 (1980)

Wiktor et al., "Protection from Rabies by a Vaccinia Virus Recombinant Containing the Rabies Virus Glycoprotein Gene", Proc. Natl. Acad. Sci. (USA), 81, 7194-7198 (1984)

Winnearls. " Recombinant Human Erythropoietin: 10 Years of Clinical Experience", Nephrol. Dial. Transplant., 13 (2): 3-8 (1998)

Wojchowski et al., "Active Human Erythropoietin Expressed in Insect Cells Using a Baculovirus Vector: A Role for N-linked Oligosaccharide", Bioch. Bioph. Acta, 910: 224-232 (1987)

Wojchowski et al, "Site-specific Antibodies to Human Erythropoietin: Immunoaffinity Purification of Urinary and Recombinant Hormone", Bioch. Bioph. Acta., 913: 10-178 (1987)

Woo, "A Sensitive and Rapid Method for Recombinant Phage Screening", Methods in Enzymology, 68, 389-395 (1979)

Wood et al., "Expression of Active Human Factor VIII from Recombinant DNA Clones", Nature, 312, 330-336 (Nov. 22, 1984)

Wright et al., "Regulated Expression of the Human-globin Gene Family in Murine Erythroleukaemia Cells", Nature, 305: 333-336 (1983)

Yajima et al., "Comparative Studies in Induction of Micronuclei by Three Genetically Recombinant and Urinary Human Erythropoietins", Mutagenesis, 8 (3): 237-241, (1993)

Yanagawa et al., "Hybridomas for Production of Monoclonal Antibodies to Human Erythropoetin", Blood, 64(2), 357-364 (Aug. 1984)

Yanagawa et al., "Isolation of Human Erythropoietin with Monoclonal Antibodies", J. Biol. Chem., 259(5), 2707-2710 (Mar. 10, 1984)

Yanagi et al., "Recombinant Human Erythropoietin Produced by Namalwa Cells", DNA, 8(6), 419-427 (1989)

Yuen et al., "The Spectrum of N-linked Oligosaccharide Structures Detected by Enzymic Microsequencing on a Recombinant Soluble CD4 Glycoprotein from Chinese Hamster Ovary Cells", Eur. J. Biochem., 192, 523-528 (1990)

Zain et al., "Nucleotide Sequence Analysis of the Leader Segments in a Cloned Copy of Adenovirus 2 Fiber mRNA", Cell, 16: 851-861 (1979)

Zieg et al., "Properties of Single-step Mutants of Syrian Hamster Cell Lines Resistant to N-(Phosphonacetyl)-L-Aspartate", Mol. Cell. Biol., 3(11): 2089-2098 (1983)

Zinn et al., "Regulated Expression of an Extrachromosomal Human .beta.-interferon Gene in Mouse Cells", P.N.A.S. (USA), 79, 4897-4901 (Aug. 1982)

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(54) TITULO DE LA INVENCION: "PROCEDIMIENTO DE CULTIVO MASIVO DE CELULAS DE MAMIFERO PARA LA OBTENCION DE ERITROPOYETINA HUMANA RECOMBINANTE Y LA ERITROPOYETINA HUMANA RECOMBINANTE OBTENIDA CON TAL PROCEDIMIENTO"

(57) RESUMEN:

La presente patente de invención describe un proceso de cultivo masivo de células de mamífero recombinantes, productoras de EPO. El proceso productivo sigue diferentes etapas de expansión, partiendo de células vialbles conservadas congeladas.
Posteriormente, se pasa a una etapa productiva en la que se utilizan medios de cultivo especialmente formulados para minimizar el agregado de suplementos proteicos.
La suplementación con insulina causa inesperadamente una elevada productividad de EPO que se encuentra con alta pureza en el medio de cultivo cosechado. Este es uno de los aspectos clave del método utilizado.
El sobrenadante de cultivo es finalmente concentrado para obtener mayor consentración de EPO en una forma apropiada para ser utilizada tal cual se obtiene o purificada ulteriormente para los usos que así lo requieran.

AR

FIGURA MAS REPRESENTATIVA Nº: